Ultrasonography Evaluation of Patency of Implanted Infra-Renal Vascular Grafts in the Rat Model.

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DECLARATION

I, *Natercia Da Silva*, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Abstract

Abstract

Introduction: Intensive research over the last six decades has resulted in minimal improvement in vascular graft development. Small animal models are the first line of species exposed to vascular graft implantation and invasive monitoring of experimental graft patency may contribute to pain, suffering, higher cost and earlier sacrifice. Non-invasive ultrasonographic evaluation of vascular implants during the conduction of animal studies allows for chronic follow-up with multiple assessments. This study aims to apply and endorse the utilization of ultrasound as a less invasive diagnostic method in determining patency of vascular grafts in units where imaging modalities like Computerized Tomography (CT) and Magnetic Resonance Imaging (MRI) are not readily available.

Methods: Pre-operative control ultrasound evaluation of the ejection fraction, aortic diameter and aortic velocity were conducted on Wistar rats (250-350g). Infra-renal aortic vascular graft implantation was then performed, with 8 rats receiving straight (1.8mm ID, 18mm length) expanded polytetrafluoroethylene (ePTFE) grafts, while 12 rats received a long (1.8mm ID, 100mm length) looped ePTFE conduit with a sealed mid-graft (10mm length) section. Ultrasonography was conducted on days 1, 3, 7 and weeks 4, 8 and 12 post operatively. Grafts were explanted if there was any ultrasonographic evidence of occlusion or at twelve-week termination of the study. Explant was preceded by angiography and followed by histological assessment of the grafts for patency.

Results: Three of the looped and all 8 of the straight grafts were patent at the 12 week explant time point, as correctly assessed by ultrasound and confirmed by angiography and histology. Three of the nine occluded looped grafts were explanted at eight weeks due to early ultrasonographic detection of occlusion; the remaining 6 were explanted at twelve weeks. There were two false positive results, which were incorrectly assessed as patent at twelve weeks of implantation on ultrasonographic evaluation, but confirmed to be occluded on angiography at explant. The results of ultrasonography evaluation of implanted infra-renal vascular grafts had a high specificity of 100% with a sensitivity of 78%. The outcome of the results between ultrasound and angiography corresponded in 18 out of 20 vascular grafts, with a calculated positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 85%.

Conclusion: Ultrasound is easily available and a non-invasive diagnostic modality allowing for safe and reliable results, which may be repeated at different time frames following vascular implants in small animal models. Ultrasonographic limitations exist, emphasizing the need for an experienced operator with adequate knowledge and training. Its use may be complicated by tortuous geometries of vessels, which is technically more challenging to evaluate with ultrasound than with imaging techniques like CT and MRI. It does, however, add information without additional loss of life or increased use of animal numbers. Ultrasound is an essential additive diagnostic tool for chronic follow-up and evaluation of vascular graft implants.

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Abbreviations

CABG	Coronary Artery Bypass Grafting
CAD	Coronary Artery Disease
CT	Computerized Tomography
D	Diastole
ECG	Electrocardiogram
HR	Heart Rate
ITA	Internal Thoracic Artery
IVC	Inferior Vena Cava
LDL	Low Density Lipoprotein
LVAW	Left Ventricular Anterior Wall
LVID	Left Ventricular Internal Diameter
LVPW	Left Ventricular Posterior Wall
MRI	Magnetic resonance imaging
NPV	Negative Predictive Value
PAD	Peripheral Arterial Disease
PCL	Polycaprolactone
PET	Polyethylene terephthalate
ePTFE	Expanded polytetrafluoroethylene
PGS	Polyglycerol Sebaceate
PPV	Positive Predictive Value
PRF	Pulse repetition frequency
PU	Polyurethane
PVA	Poly vinyl alcohol
PWV	Pulse wave velocity
S	Systole
SAVC	South African Veterinary Counsel
GSV	Greater Saphenous Vein
TEV	Tissue Engineered Vessel
UCT	University of Cape Town

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Section 1: Literature Review

1. Introduction

Ultrasound is a non-invasive diagnostic tool which has seen great advances since its development in the mid twentieth century.(1, 2) Ultrasound imaging was first used in animals in the 1980s, when grey-scale ultrasonic imaging could view the reproductive organs and cycles in horses and cattle.(3, 4) The improvements in ultrasound technology have led to the progression from large bulky machines with suboptimal imaging, to portable, sophisticated, diagnostic instruments which combine elements of physics, engineering, physiology and medicine into a single functional imaging modality.(1) Due to the non-invasive nature of ultrasonography as an imaging technique and its ease of use, it is now widely used in a great diversity of modalities and applications and has expanded and revolutionized research in animals. (3, 5)

Small animal models are considered to be the low cost and high throughput model of choice in the initial in vivo analysis of vascular grafts.(2, 6) Pre-clinical research aims to develop vascular graft materials that mimic the structure and function of autologous vessels.(7) These grafts may be of use in human recipients who do not have sufficient autologous conduits to treat coronary artery disease, peripheral vascular disease or to provide vascular access for renal dialysis.(7, 8) Invasive methods may be used to determine patency of experimental vascular grafts in animals, but this has significant consequences.(6, 9, 10) Invasive monitoring in animal research may contribute to pain, suffering, higher cost and earlier sacrifice of animals due to humane end points being reached.(6, 10) Non-invasive ultrasonographic imaging to determine patency of vascular graft implants in animal studies, allows for long term follow-up where multiple evaluations may be needed.(10, 11) Limited research has been published on use and efficacy of various modes of ultrasound in the evaluation of vascular graft patency in rodent models.(8, 12-17) Vascular graft development is an ongoing field of research and these novel techniques of neoartery formation with biodegradable tissues and in vitro studies, take time and prolonged experimental implantation in animal models.(7) Therefore ultrasonography may be used as a less invasive diagnostic imaging modality, to assess patency of these grafts in vivo, adding substantial knowledge to this field of research.

2. Clinical Vascular Conduits

Cardiovascular pathology is one of the leading causes of mortality and accounts for 30% of deaths world-wide, of which coronary artery disease (CAD) caused by atherosclerosis, is the highest underlying factor.(18, 19) There are a wide variety of biological vascular conduits available for coronary artery bypass grafting (CABG).(18, 20, 21) The most commonly used arterial conduits are the right and left internal thoracic and radial arteries, less commonly the right gastroepiploic artery and occasionally the ulnar artery, splenic artery, and inferior epigastric artery may also be used.(20-22) The greater saphenous vein (GSV) is the most abundantly used venous conduit.(23-25)

Peripheral arterial disease (PAD) involves arteries distal to the heart, supplying the legs, stomach, arms and head, which are narrowed due to atherosclerosis, leading to decreased tissue perfusion.(23, 24) Vessels to the lower limbs are most commonly targeted and large calibre arteries, such as aorto-iliac disease, favour prosthetic grafts for reconstruction of the vessel and reconstitution of distal flow.(23) Saphenous veins or commercially available synthetic grafts function well in these anatomical regions.(21, 23, 25) Prosthetic graft performance as small vessel conduits are poor.(7, 25, 26) This include the regions found in infra-inguinal bypass, where vessel diameters are less than six millimetres.(6, 24, 25, 27)

The global burden of CAD and PAD is worsened by the increased rise of diabetes mellitus, leading to target organ damage and additionally adding to the amount of patients that develop end-stage renal disease.(7, 26, 28) . This disease risk profile adds to the higher numbers of surgical procedures performed to establish a vascular access port allowing for haemodialysis.(7, 29) Arteriovenous vascular access in dialysis patients, are prone to develop complications such as infection, pseudoaneurysm formation and occlusion due to thrombosis, to name but a few, requiring repeat procedures and additional vascular conduits.(7, 30)

The abovementioned medical conditions contribute to the significant vascular burden of disease in society and require innovation and continuous pursuit of novel vascular grafts. The ideal bypass conduit in these haemodynamic demanding environments, are arterial conduits.(7, 20, 25) Arterial conduits have an antithrombotic endothelial surface but unlike CABG's, due to lack in length, arterial autografts in PAD are not a viable solution and therefore venous conduits are used.(6, 23, 24) As mentioned, the GSV is the main conduit used for both small and medium distal vascular bypass procedures.(23, 25) Prosthetic vascular conduits have good outcomes in vessels with larger diameters, lower resistance and higher flow rates.(21, 23, 25) When a high quality vein is not available, synthetic material used as vascular conduits up to the level of the popliteal artery, have acceptable outcomes. (23, 25)

Patency of prosthetic grafts below popliteal region, however, have been unsatisfactory.(23, 25)

Research has now turned its focus to attempt mimicking the properties of arterial vessels with various graft material (e.g. tissue engineering), different graft reconstruction methods (e.g. electrospinning) and impregnation techniques with various substances (e.g. heparin) in an attempt to develop improved small vessel grafts that deliver long term patency.(26, 31-34) When a biological graft, such as a venous or arterial graft described above is not available, artificial grafts are used.

3. Artificial Vascular Conduits

3.1 Synthetic Grafts

Expanded polytetrafluoroethylene (ePTFE, Goretex ®) and textile polyethylene terephthalate (PET, Dacron®) grafts,(21) are synthetic alternatives to autologous grafts, both having low success rates as small diameter (< 6mm) vascular replacements.(7, 35) They are the most abundantly available and used synthetic materials with no evidence of superiority of one material over the other.(7) The patency rate of these synthetic materials as conduits in large diameter, high flow vessels (>6mm) is equal to that of saphenous vein grafts.(7) Patency declines and these grafts serve as poor conduits in small vessel disease due to thrombogenicity and intimal hyperplasia.(25, 32, 35, 36) They have patency rates of only 25-40% at 5 years as femoral-popliteal conduits compared to 70% rate of patency found in reversed saphenous vein conduits.(7, 23, 35) There are essentially no non-autologous conduits with acceptable patency rates that can be used in the coronary circulation or peripheral small vessel bypass.(23, 27) Synthetic arteriovenous grafts used as vascular access for haemodialysis have very poor success rates.(30, 37)

3.2 Experimental Grafts

Various studies have revealed that polymer nanofibers seem promising for the use in fabrication of small-diameter vascular scaffolds.(7, 37-41) Numerous novel synthetic fibres are investigated as feasible alternative materials to be used in vascular graft reconstruction for small diameter vessel bypass. These materials include polycaprolactone (PCL), polylactide, bacterial nanocellulose and poly vinyl alcohol (PVA).(39, 40, 42) Our group has developed grafts based on the polymer polyurethane (PU).(21, 22) PU consists of three monomers, namely isocyanate (crystalline), polyol (amorphous) and a chain extender.(21, 43) This allows the material to accept a range of physical properties from soft and elastic, to hard and rigid.(21, 43) PU is generally degradation resistant but by adding a certain polyester as

the polyol, it becomes biodegradable and allows for faster endothelialization and less intimal hyperplasia, currently making it an appealing experimental conduit for small diameter vascular access.(43, 44)

3.3 Biodegradable Synthetic Scaffolds

The initial attempt to develop a tissue engineered vessel (TEV) replacement in vitro, was recorded in the late nineteen hundreds and this research became the stepping stone of further developments.(45) Biodegradable scaffolding is the process of tissue engineering which combines a degradable polymer with biologically active material to construct a composite matrix.(31, 32) Polymers give structural stability to the biological materials and gels act as a delivery system for biologically activated substances.(46) The vascular graft is fabricated from a biodegradable polymer. The polymer slowly degrades giving the seeded cells time to form an extracellular matrix.(32) Tissue engineered vessels need to be transplantable and withstand circulatory pressures without adverse formation of aneurysm or rupture with leakage.(27) Vessels produced need to be free of immunological degradation once implanted and have anti-thrombogenic properties. (27) These engineered vessels need to be rapidly reproducible at clinically relevant scales.

3.4 Biological Scaffolds

This process makes use of decellularization and recellularization matrices in the absence of synthetic tissue to copy the extracellular structure of a vessel.(32) The scaffold promotes the ingrowth of fibroblasts and smooth muscle cells from adjoining tissue into a cellular depleted matrix after implantation.(32, 47, 48) This cellular depleted matrix gradually reconstructs itself from the allograft into an autogenous, self-renewed life bearing tissue.(32) Methods of decellularization regimens keeps the microvascular basement membrane of the extracellular matrix scaffold intact, maintaining the structure of the vessel so that recellularization can occur on the fixed matrix(27, 32) The use of gel-based endothelial recruitment methods seem to be the most promising, along with the decellularization-recellularization strategies, in an attempt to achieve microscopic vascular perfusion of TEV.(27, 32) In addition to the above methods, the era of microfabrication of vessels are also in early stages of development and use.(47)

To overcome the difficulties entailed by the transplantation of autologous vessels or synthetic conduits, tissue engineering and scaffold generation procedures represent newer techniques to attempt to create living conduits with features similar to autologous vessels.(26, 33, 48) These novel techniques of neoartery formation with biodegradable tissues and in vitro studies, take time and prolonged experimental implantation in animal models are required.(7)

4. In Vivo Animal Implants

With the development of medical devices or conduits, translation to clinical implantation in humans is preceded by animal models. In order to resemble and mimic human anatomy, pathology and physiology, a living functional cardiovascular system is needed.(49) This is particularly crucial in vascular research to asses flow and thrombogenicity.(21, 49) Animal models are cheaper, easily available, and can develop a pre-programmed desired pathology as well as mimic the vascular physiology of humans.(49) No ideal animal model exists to assess the various parameters and physiological stressors needed in the preclinical research of in vivo vascular conduit experimentation.(2, 49) Different species and strains are used to assess various variables in the methodology and function of vascular graft development.(6, 49)

Mice are the ideal animals used in assessing molecular pathways, due to the ease of access of varying populations of knock-out species.(34, 49) However, mice are not ideal for the use in vascular graft models, due to their small size, which makes the length of implantable grafts suboptimal.(34) Rats and rabbits, slightly larger rodents, allow for longer vascular implants and are therefore more suitable models for proof of concept studies, but are in turn limited by not having the knockout effect.(34) Rabbits are superior to rats in assessment of vascular thrombogenicity, due to greater similarities in haemostatic mechanisms such as thromboplastic and fibrinolytic properties, when compared to humans.(49) An additional similarity to humans, rabbits also resemble endothelisation of vascular conduits in the perianastomotic regions.(43, 49) Rabbits, however, are disadvantaged by having an increased risk of developing paraplegia after infra-renal aortic clamping for placement of aorto-iliac conduits.(34) They also present with a wide variation in patency of vascular grafts in various implantation locations, presenting with difficulties in finding consensus when determining the region of implantation.(49)

Larger animal models, including sheep and pigs, are more appropriate in relating to human vasculature due to the similarities in size of vessel and shear stress of vessel walls.(34, 49) Other large species have significant variability when compared to humans, especially with regards to the coagulation cascade.(49) The former holds true in the canine model, where studies have shown a tendency towards a hypercoagulable state, with increased platelet aggregation, clearly not being an ideal feature in vascular graft studies.(49-51) Another concerning feature is the high patency rate and rapid endothelialization of vascular synthetic grafts in dogs with little or no signs of intimal hyperplasia, which does not translate into clinical

findings in humans.(25, 34, 43) Dogs, though easy to handle, are therefore a poor model to use in vascular studies.

The pig model is known for the responsive nature of its vasculature and is at present the preferred breed for in vivo experimentation of balloon angioplasty techniques and coronary stenting.(49) It is ideal for coronary studies, to assess the rate of restenosis of vessels but may also be useful for research of more peripheral vasculature such as the carotid arteries (8, 11) In contrast to humans, baboons, dogs and pigs are all able to develop an endothelial layer on the luminal surface of an arterial conduit.(25, 34) Non-human primates are the most accurate vascular model, being most similar to humans, even in terms of coagulation, but high cost, and ethical concerns limit their use in laboratory research.(25, 49) There is variation in the amount of intimal hyperplasia that develop during vascular graft studies in animals and an increased intimal response is most pertinently observed in pigs.(49) There is less of a intimal response noted in primates and rabbits and intimal hyperplasia is almost non-existent in canines.(49) Therefore the pig model is known to develop stenosis of vessels six times faster than in humans, and is therefore a poor model to assess long term patency of vascular grafts.(34)

Sheep, in turn, have high vascular occlusion rates but restrict endothelisation only to regions around the anastomoses of the graft, thus having similarities to the human physiological reaction to vascular conduits.(34, 49) This makes it the preferential animal of choice for research involving physiological mechanisms of vascular graft implantation.(49) It does present some differences of thrombogenicity when compared to humans, and has an increased rate of calcification.(25, 49) Sheep are the large animal vectors of choice to use in vascular graft research particularly involving vessels larger than six millimetres in diameter.(34, 49)

Rodents like rats and rabbits are the small animals of choice used in vascular graft experiments.(49) Rabbits are preferred in experiments with vascular conduits up to four millimetres in diameter. (49) They also present with the advantage of allowing for bilateral implantation of conduits due to longer vessels in their hind legs.(34, 49) Rabbits have a greater similarity than rats to humans with regards to physiological parameters such as the coagulation cascade, the extent of endothelialization and rate of patency of vascular conduits.(34, 49) The difficulty in the smaller rat model arises with perianastomotic endothelisation, which presents as a problem in grafts limited to 10mm in length.(49) Conduit lengths of at least 20mm is required to avoid endothelisation resulting from mere perianastomotic ingrowth but this length of implant is rarely possible in these models.(49) Our unit has overcome this difficulty by looping the infra-renal vascular graft allowing implantation

of grafts up to 100mm in length.(22) Controversially, rats can be used to assess intimal proliferation, which is magnified in this model, and is furthermore used to assess strength and healing of vascular graft conduits.(22, 49) The advantages of rats in vascular graft research is that they have systolic blood pressures similar to that of humans, exposing the graft to adequate pressures and the faster heart rates allow for multiple cycles of high pressure flow.(21) Rats are also easy to house and handle, readily available and are considered as an adequate low-cost, high throughput model making them ideal for multiple vascular graft implants in early graft assessments.

5. Ultrasonography

5.1 History

Ultrasonography is a diagnostic tool widely used in visualizing soft tissue and organs in both medical and veterinary fields.(3) Ultrasound produces images as sound waves come into contact with tissues of varying density.(52) The earliest documented application of ultrasound was introduced by Dussik in 1942. (1) The first application of this modality involved imaging the brain, the abdomen for gallstones, obstetric use and eye assessment in ophthalmology. (1, 53) Initial examinations were made using A (Amplitude) Mode with patients immersed in water.(1) Contact ultrasound with transducers directly applied to the patient's skin, was first developed in Glasgow in the 1960's.(1, 53) The sound waves produced by a transducer, pass through tissues of varying densities with different acoustic impedance.(6, 52) The different densities of tissue that the sound wave encounters is recorded as an "acoustic impedance mismatch",(52) and these sound waves are conducted back and interact with crystals on the ultrasound transducer.(3, 36, 52) This produces a current of electrical signals interpreted through computational analysis, processed and displayed on the screen.(6) This allowed for the development of B (Brightness) Mode which produces a grey-scaled, two-dimensional cross sectional image in real time. Moving structures can be analysed due to the development of real time imaging using M (Motion) Mode ultrasonography.(1, 2, 54) The assessment of blood flow, however, has only been made possible with Doppler ultrasonography where velocity of moving tissue is determined.(6)

5.2 Small Animal Models

In the premillennial era, ultrasonographic imaging in laboratory research was restricted to large animals.(4, 6, 55) Ultrasound imaging techniques in animals have been adapted from human

diagnostics methods, but there is an increased demand for improved spatial and temporal resolution in imaging of smaller animals due to the limitation in their size and high physiological demand with extremely rapid heart rates.(2, 6, 10) Developments in ultrasound imaging has led to commercially accessible, advanced ultrasound systems with transducers that accurately interpret spatial and temporal resolution to acquire adequate imagery of vascular structures of rats and mice.(2, 6, 10)

In rodents both increased spatial (consisting of axial and lateral) resolution and temporal resolution is essential for adequate imaging.(6, 10) Axial resolution is defined by the proficiency in determining the presence of two detached but closely related structures lying parallel to the ultrasound beam.(6) Axial resolution is amplified with sound waves of increased frequencies.(6) Lateral resolution is defined by the ability to determine that two closely related structures are separate, lying perpendicular to the beam, a parameter which improves by narrowing the ultrasound beam width.(6) Both these parameters increase in accuracy with higher frequencies emitted from the transducer.(6, 52) Temporal resolution alludes to the capability of ultrasound to discern between two events at differing time frames and is calculated as the amount of image frames acquired per second.(6) Temporal resolution plays an important role in imaging moving structures like the heart in small animals with very fast heart rates.(6, 10) The limitation of higher frequency probes is that the higher the frequency of the probe, the lower the penetration depth of the sound wave, therefore giving a clearer image by improving lateral and axial resolution but making deeper structures difficult to visualize.(6) This, however, does not affect the transducer selection in small animals, since transducers with frequencies of up to 10MHz can penetrate as deep as 50mm, which is an adequate depth of penetration in rodents.(6, 10)

The difficulties in conducting ultrasound evaluation in small animals versus large animals are clearly defined.(2, 6) Limitations in large animal screenings are related to the depth of imaging, with most transducers not having adequate penetration depths beyond 25cm.(2) In small animals the depth of imaging is less of a problem, but difficulties arise in separating various tissue planes. Faster heart rates, the need for anaesthesia and the decrease in body temperatures, which may result in haemodynamic instability during ultrasonography, also present with additional difficulties in small animals which must be closely monitored during the procedure. (56)

5.3 Vascular Ultrasonographic Imaging

Doppler ultrasonography is a less invasive diagnostic technique used in determining vascular flow and patency of vessels in humans and animals.(10, 11, 52) The direction of blood flow and velocity is determined on spectral and colour flow Doppler modalities by using the "Doppler shift principle".(6, 10, 52) The variation in sound wave frequencies that ensues when the waves are reflected by mobile objects, including blood cells, is described as the "Doppler Effect".(6, 10) The discrepancy between the transmitted and reflected sound wave frequency is termed the "Doppler Shift".(6, 52) Spectral Doppler consists of pulse wave and continuous flow Doppler ultrasound.(6, 52) Pulse wave Doppler displays velocity measured at a single point whereas Continuous flow Doppler allows you to measure velocity continuously and dynamically along an entire line.(6, 52) Colour flow Doppler in turn, displays the route of the blood flow and the velocity of flow in a vascular structure in real time and can be used to evaluate turbulence in vessels.(6, 8, 10) Doppler ultrasonography is particularly useful in assessing the flow characteristics within vascular structures, including the heart. It is affected by the direction of the flow of blood in association with the ultrasound beam and may erroneously attenuate flow measurements if the ultrasound beam is not parallel to blood flow.(10) Higher resolution Doppler imaging modalities have become a popular technique in assessing vascular physiology.(10, 56)

5.4 Application of Vascular Imaging in Laboratory Research

Doppler ultrasonography has been used in determining flow in vascular grafts as early as 1981, where an assessment in patency and flow of PTFE vascular grafts in humans was facilitated by Doppler blood flow measurements.(57) In this earlier study, PTFE grafts did not transmit the ultrasound beam adequately to evaluate flow within the graft.(57) It was thus erroneously concluded that Doppler ultrasonography measurements over PTFE grafts were not detectable and ultrasound could not reliably be used to assess flow in the early post-operative periods.(57) This study, however, was soon followed by others, where Doppler was able to accurately differentiate between ligated femoral arteries in rats and ePTFE interposition grafts up to one millimetre in size.(58) It allowed for a 100% accuracy at 20 days of implantation, but this accuracy did, however, decrease at 24 weeks.(59)

In similar research conducted in large animal models, inaccuracy of Doppler findings seems to be less of a problem. Doppler ultrasonography was found to be more accurate when used to evaluate patency or occlusion of implants in carotid arteries in pigs at different time frames, compared with the gold standard, namely angiography.(9, 11) At four week follow-up in this

study the negative predictive value (NPV) was still as high as 95%, positive predictive value (PPV) of 92%, sensitivity of 86% and specificity of 92% in determining patency with ultrasonographic imaging of carotid grafts in pigs.(11)

The use of Doppler ultrasonography in assessing the stenosis and flow velocity in carotid arteries is common and has been used in small animal models as well. Doppler ultrasonography has been utilized in diabetic Long-Evans rats, to determine common carotid arterial blood flow velocity and colour Doppler sonography, detecting significant carotid artery stenosis.(14, 16) Various studies have been reported to use Duplex Doppler modalities in accurately assessing the level and severity of carotid artery stenosis in rats.(16) Accurate assessment of the level of occlusion, with stenosis ranging between 30-85% of the diameter of carotid arteries of rats could be detected and evaluated.(16) Colour and pulsed wave Doppler modalities are utilized to determine flow turbulence, M-mode measures the vessel cross-sectional dimensions and B-mode is used to determine vessel length.(15) Ultrasonography was able to assess three intricate graft manipulations in carotid vein grafts in mice, with turbulence, cross sectional dimensions and length of vessel measured with above modalities.(15) All results were accurately reproducible when compared to histology ex-vivo.(15)

The first reported ultrasonographic imaging of abdominal aortic aneurysms in mice, was recorded in 2001.(8) There has been significant development of ultrasound imaging modalities since, and ultrasonography has been used to evaluate progression of abdominal aortic aneurysms in rats.(8, 60) Measurements including assessment of vessel stiffness and diameter of the aneurysmal segments of the aorta can be detected on Doppler imaging.(13) Pulse wave Doppler is used to determine changes in velocity and high-frequency ultrasound can be used to characterize abdominal aortic aneurysm progression and thereby contribute to increased understanding of aneurysm pathogenesis.(8, 60) Due to the progression and development of high frequency ultrasound images that provide better spatial resolution, it has been possible to accurately quantify and measure aortic diameter and wall thickness in mice and rats. (8) Transducer probes are continuously improving and more intricate parameters such as heart rate, peak ascending aortic velocity, aortic diameter, left ventricular ejection fraction and cardiac output can be accurately determined. (56, 61, 62)

In the past five to ten years there have been developments with regards to ultrasonographic technology in animal research, especially small animal models. Ultrasound is an attractive diagnostic modality since it allows for long term follow up, without additional harm to animals. However, in studies conducted as recently as 2018, it is still only used as one of three modalities to determine patency in vascular graft models.(42) The gold standard in

determining patency still accepted as angiography, followed by histological assessment.(9) Even in studies with MRI and CT angiography available, both modalities could only confirm patency at explant.(42) These imaging modalities are not readily available in all research units. Even though Doppler ultrasonography is utilized in vascular graft studies as an imaging modality, it is not used to determine time of explant of grafts. Patency is only confirmed by invasive measures during termination of the study at explant.

The implantation of small-diameter electrospun interposition grafts in the rat aorta, were assessed for patency at one and three months by using only high frequency colour Doppler ultrasonography.(6, 39) Assessment of flow velocity at proximal, distal and the centre of the graft was made. Ultrasound adequately indicated patency of all grafts implanted and this was confirmed on histological assessment. No mention of angiographic evaluation was made. All grafts were deemed patent at three months with only the use of Doppler ultrasonography and confirmed on histological assessment after explant.(13, 39)

In similar studies with silk interposition grafts implanted in the female rat aorta, patency was also only assessed with Doppler ultrasonography and obstruction of grafts were detected on Doppler ultrasound.(44, 63) The presence of aneurysm formation, thromboses and aortic diameter was recorded.(8, 44) The occluded grafts were evaluated with ultrasonography and confirmed to be occluded prior to explant with a hundred percent specificity and sensitivity for detection of graft occlusion and patency on ultrasonography.(13, 44)

6. Summary

Our unit has conducted studies looking at transmural ingrowth in implanted vascular grafts in rat models. (21, 43) We have found that by looping the graft, one achieves a broad endothelial surface to assess transmural endothelialization more accurately over longer time frames than when compared to straight grafts.(21, 43) Although technically more challenging, making use of a loop graft instead of standard straight implants allows for more length so that the anastomoses is at a distance from the isolated mid-graft segment. Results of studies previously conducted, validated the loop graft to be a high output model for evaluating the isolated healing of the mid-graft segments in vascular conduits.(43) Due to advances in ultrasound imaging technology, it is being used more frequently as a diagnostic tool and we would hope to use this modality in the assessment of occlusion or patency in our complex infra-renal loop graft models.

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Section 2: Manuscript

Ultrasonography Evaluation of Patency of Implanted Infra-Renal Vascular Grafts in the Rat Model.

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Abstract

Introduction: Intensive research over the last six decades has resulted in minimal improvement in vascular graft development. Small animal models are the first line of species exposed to vascular graft implantation and invasive monitoring of experimental graft patency may contribute to pain, suffering, higher cost and earlier sacrifice. Non-invasive ultrasonographic evaluation of vascular implants during the conduction of animal studies allows for chronic follow-up with multiple assessments. This study aims to apply and endorse the utilization of ultrasound as a less invasive diagnostic method in determining patency of vascular grafts in units where imaging modalities like Computerized Tomography (CT) and Magnetic Resonance Imaging (MRI) are not readily available.

Methods: Pre-operative control ultrasound evaluation of the ejection fraction, aortic diameter and aortic velocity were conducted on Wistar rats (250-350g). Infra-renal aortic vascular graft implantation was then performed, with 8 rats receiving straight (1.8mm ID, 18mm length) expanded polytetrafluoroethylene (ePTFE) grafts, while 12 rats received a long (1.8mm ID, 100mm length) looped ePTFE conduit with a sealed mid-graft (10mm length) section. Ultrasonography was conducted on days 1, 3, 7 and weeks 4, 8 and 12 post operatively. Grafts were explanted if there was any ultrasonographic evidence of occlusion or at twelve-week termination of the study. Explant was preceded by angiography and followed by histological assessment of the grafts for patency.

Results: Three of the looped and all 8 of the straight grafts were patent at the 12 week explant time point, as correctly assessed by ultrasound and confirmed by angiography and histology.

Three of the nine occluded looped grafts were explanted at eight weeks due to early ultrasonographic detection of occlusion; the remaining 6 were explanted at twelve weeks. There were two false positive results, which were incorrectly assessed as patent at twelve weeks of implantation on ultrasonographic evaluation, but confirmed to be occluded on angiography at explant. The results of ultrasonography evaluation of implanted infra-renal vascular grafts had a high specificity of 100% with a sensitivity of 78%. The outcome of the results between ultrasound and angiography corresponded in 18 out of 20 vascular grafts, with a calculated positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 85%.

Conclusion: Ultrasound is easily available and a non-invasive diagnostic modality allowing for safe and reliable results, which may be repeated at different time frames following vascular implants in small animal models. Ultrasonographic limitations exist, emphasizing the need for an experienced operator with adequate knowledge and training. Its use may be complicated by tortuous geometries of vessels, which is technically more challenging to evaluate with ultrasound than with imaging techniques like CT and MRI. It does, however, add information without additional loss of life or increased use of animal numbers. Ultrasound is an essential additive diagnostic tool for chronic follow-up and evaluation of vascular graft implants

1. Introduction

Ultrasound includes any sound frequency above the human audible range (20kHz) and the process of ultrasound imaging utilizes a transducer containing piezoelectric material which vibrates.(1, 2) These vibrations emit high-frequency sound waves, colliding with tissue as an acoustic interface.(3) A portion of the sound wave is reflected back as a measurable voltage detected by the transducer.(1, 2, 4, 5) If the tissue interface that the sound wave collides with consists of two varying densities, different voltages will be reflected back and this phenomenon is known as an "acoustic impedance mismatch".(2, 3, 6) Diagnostic ultrasonography relies on the "Pulse-Echo principle",(2, 7) where measuring the time between the transmission of the impulse and the reception of a given echo signal, allows for the construction of a two dimensional anatomical image.(1, 2) By knowing the speed of the sound and time taken for the sound waves to return, the depth of the tissue the echo is reflected from, may be calculated.(2, 7) This input is converted to interpretable images on the monitor through a computational algorithm.(2) Ultrasonography allows for the interpretation of tissue structures, flow dynamics and function, and has been used for decades in clinical medicine and is now more often being applied to research in the laboratory setting.(1, 7)

Ultrasound imaging methods applied in small animal research have been adapted from clinical human diagnostic applications.(7) The faster heart rates and smaller anatomy in rodent models increase the demand for the better quality of spatial and temporal resolution.(3, 7) Imaging techniques and equipment have been modified and enhanced to meet the demands of these higher-resolution requirements.(3, 7, 8) There is an improvement in spatial resolution with an increase in frequency and higher frequency transducers have been produced, which has allowed for accurate quantification of the vascular dimensions in mice and rats.(3, 9)

The "Doppler Effect",(2, 3) is used to describe the motion of the sound impulse which is reflected from a moving target.(3) This impulse is deflected either towards or away from the transducer, with higher frequencies observed from structures moving towards the transducer and lower frequencies from those moving away, this is known as the "Doppler Shift Principle".(3, 7) Doppler signals can be displayed as colour Doppler with "colour flow mapping" or be presented as "velocity profile over time" known as spectral Doppler.(2) Higher resolution Doppler systems are frequently used in evaluating human and animal vascular physiology.(7, 10)

This diagnostic modality applied in animal research, influences laboratory studies involving vascular conduit production, allowing non-invasive and continuous monitoring of progress without early or unnecessary sacrifice of animals.(9, 11) Small animal models are considered to be the low cost and high throughput model of choice in the initial analysis of vascular grafts. Pre-clinical research aims to develop vascular graft materials that mimic the structure and function of autologous arteries.(12) These grafts may be of use in human recipients who do not have sufficient autologous conduits to treat coronary artery disease, peripheral vascular disease or to provide vascular access for renal dialysis.(6, 12)

For the past six decades, intense research has been conducted in an attempt to create small diameter vessel conduits that translate into clinically acceptable autologous, vascular substitutes.(12, 13) Expanded polytetrafluoroethylene (ePTFE, Goretex ®) and textile polyethylene terephthalate (PET, Dacron®) grafts, (14) are alternatives to autologous grafts. Both these synthetic materials have high failure rates when used in narrow diameters (< 6mm) as small vessel replacements.(12, 15)

Our laboratory has previously shown that straight infra-renal ePTFE grafts have confirmed long term-patency, whereas looped grafts with non-porous test segments are more prone to early occlusion.(16) Invasive methods may be used to determine patency, but these have significant consequences.(9, 11) In animal research, invasive monitoring may contribute to pain, suffering, higher cost and earlier sacrifice of animals due to humane end points being reached.(9) Non-invasive use of ultrasonography during vascular implants in animal studies allows for chronic evaluation and follow-up of these models.(9) Assessment of the accuracy of implementation of ultrasound evaluation of our unit's vascular grafts, could add additional information to vascular research conducted and a means of refinement and reduction of animals used in our graft studies.

2. Materials and Methods

2.1 Study Design

The protocol was approved by the Animal Ethics Committee (AEC) of the University of Cape Town (UCT) (protocol AEC 017008) and performed in compliance with "*the Guide for the Care and Use of Laboratory Animals*, *Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council and ARRIVE guidelines*. The surgical procedures and ultrasound investigations were performed by personnel accredited by the South African Veterinary Council (SAVC).

Ultrasonography was performed on Wistar rats (250-350g). A control group (n=10) underwent pre-operative ultrasonographic assessment of the aorta and heart to measure abdominal aortic flow, flow velocity, aortic diameter and ejection fraction. The experimental phase consisted of 20 Wistar rats divided into two groups for infra-renal aortic vascular graft implantation. Group 1 (n=8) received an 18mm length of ePTFE straight graft implant. Group 2 (n=12) received a 100mm looped graft implant consisting of a 90mm ePTFE conduit with a 10mm sealed mid-graft polyurethane section.

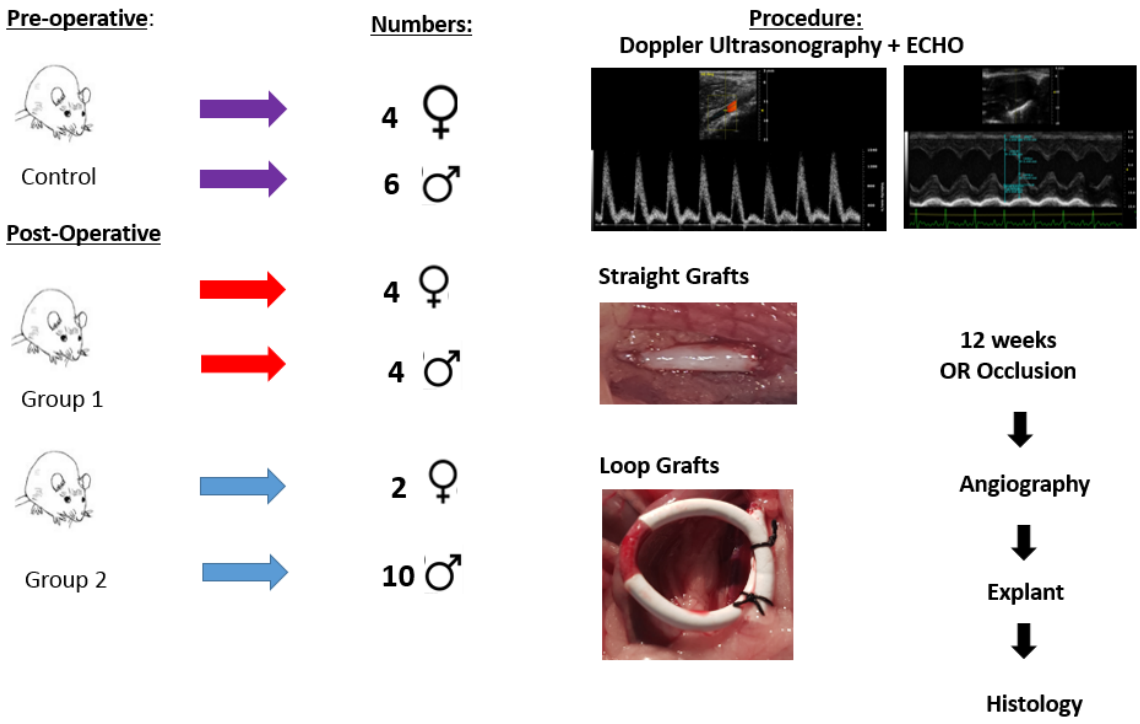

Figure 1: Schematic Presentation of Methodology

2.2 Graft Production

2.2.1 Polyurethane (PU) Mid-Graft Segment Construction

The mid-segment of the loop graft was constructed as previously described.(14) Briefly, a porous cylindrical cast was produced by tightly stacking gelatin beads in a glass mould around a centrally located mandrel and applying a 10% PU solution. The remaining voids were filled by providing a driving pressure (750 kPa) to the solution with an additional downstream external vacuum (100 kPa).

The cylindrical casting was demoulded from the tube, and the polymer phase inverted for 24 hours at room temperature with 96% ethanol.(14) The gelatin beads and additional solvent were washed from the polyurethane in a water solution at 60 degrees Celsius over five days, and dried to produce a tube with well-defined interconnected porosity.(14) The resulting cylinders were cut into 1cm segments and used to form the mid-portion of the loop graft.

2.2.2 Loop Conduit Construction

A 33 cm long nylon cord was tightly wound around a plastic rod (1.5mm [OD]) and secured with cable ties and subsequently placed in boiling water for six minutes.(14) Following rapid cooling, the cord was unravelled in its heat-set form and then cut into 11 cm segments. Each curved segment was used to construct a loop graft. A one centimetre bevelled PU mid-graft segment (described above) was placed in the centre of each 11 cm nylon cord. Two ePTFE graft segments measuring 4,5cm in length, were fed onto the nylon cord and glued to either end of the bevelled PU mid-graft segment.(14) This construction formed a 10cm looped graft. The abluminal surface of the PU mid-segment of each looped graft was coated with a 10% solution of PU/chloroform as a sealant, to form an impermeable layer.(14)

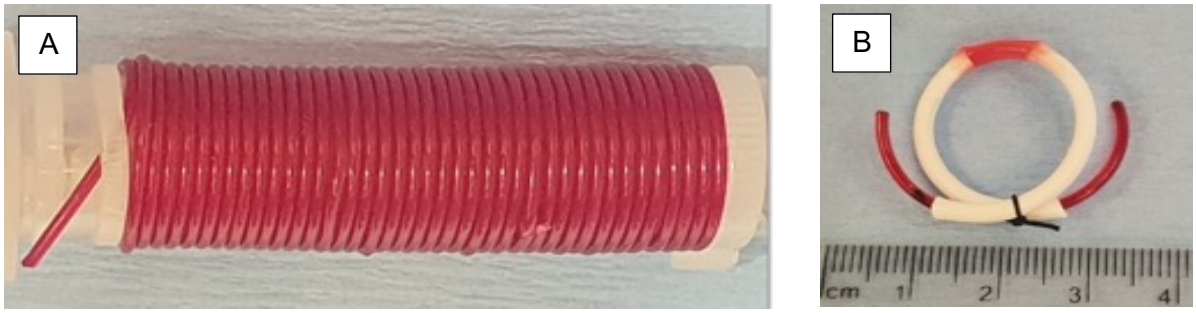

Figure 2: Loop Graft Construction *(A) Displays a 33cm long nylon cord (1.5mm [OD]) wound tightly around a 15mm cylindrical rod and secured with cable ties. (B) The nylon cord is divided into 11cm long segments with a mid-graft PU segment fed onto the nylon cord and the two ePTFE graft segments glued to either side of the PU segment. The PU segment is sealed with thin coat of PU/Chloroform.*

2.3 In Vivo Implantation of Grafts

An inhalational mask induction anaesthesia with Isoflurane (5%) at 1.5l/min was used. A midline laparotomy was performed under strict aseptic conditions and the infra-renal aorta was dissected to display the left renal artery cranially and the aortic bifurcation with iliac vessels caudally. The iliolumbar branches were cauterized, aorta mobilized and heparin (1mg/kg) was injected via the iliolumbar vein. Following proximal and distal clamping, the infra-renal aorta was transected in a bevelled fashion, without excision allowing for enough aortic length for manipulation of the straight and the loop-grafts segments during implantation.

Graft conduits were cut to the required size and a thin arterial guidewire (Arrow® Seldinger Arterial Catheter, North Carolina, US), was introduced through the graft lumen into the proximal or distal lumen of the aorta. The guidewire was held in place temporarily in the proximal and then distal aortic lumen with an arterial clamp, to separate anterior and posterior vessel walls. This technique was used as a preventative measure to avoid suturing both arterial walls together during the anastomoses, which would lead to the obstruction of the vessel lumen. The guidewire was removed once four sutures had been placed, one at each corner of the graft proximally and then distally, ensuring a patent lumen. Once the guidewire was removed the anastomoses of the graft to proximal and distal abdominal aorta were completed with 9-0 nylon (Johnson & Johnson, New Brunswick, NJ), in a total of ten to twelve interrupted sutures.

The graft was de-aired by first releasing the distal clamp, filling the graft with blood, followed by the slow release of the proximal clamp. The anastomotic sites were compressed until haemostasis was achieved. Looped grafts were buried in the retroperitoneal space. A patency test of the graft was performed under vision with a distal compression technique and ensuring both hind limbs of the rat was well perfused before closure. The midline laparotomy was closed with 2-0 Ethibond (Johnson & Johnson, New Brunswick, NJ) sutures in layers. During the recovery phase, the rats were isolated in separate cages for the first 24 hours and given softened food and water. Once recovered they were transferred back to cages with fellow rats for social interaction. Pain was controlled post-operatively with subcutaneous injections of buprenorphine (0.05 mg/kg) three times daily for 72 hours, or until no features of pain could be detected.

2.4 Ultrasound Examinations

Ultrasonography was conducted by two of the researchers (NDS; NH). All the results were cross-evaluated by both to standardize findings. This procedure took place on day 1, 3, 7, week 4, 8 and week 12 post operatively. A single dose of buprenorphine (0.05mg/kg) was administered as premedication 30 minutes prior to induction for Ultrasonography. The animals were induced with isoflurane at 5% in a transparent anaesthetic chamber and while spontaneously breathing, maintained on 1.25% on nose cone after being transferred supine onto a heated platform with integrated electrocardiogram, allowing for heart rate, breathing rate and temperature monitoring.

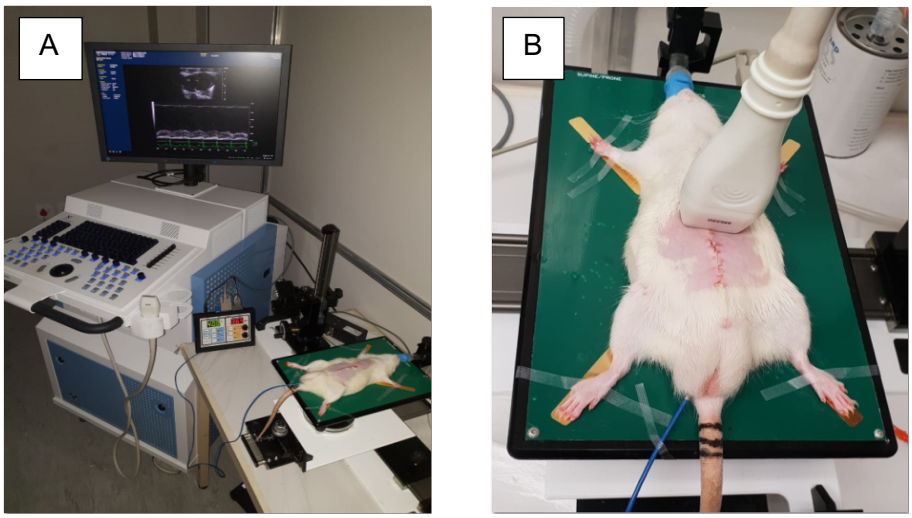

Figure 3: Ultrasonography Setup *(A) Vevo 2100 ® with heated platform and respiratory monitor (B) Rat induced with isoflurane, in a supine position on a heated platform with integrated electrocardiogram, ultrasound probe and temperature probe.*

The area of incision was carefully shaved with an electric razor. Hair removal was further achieved with dilapidating cream (Nair® Hair Removal Cream, New Jersey, US) applied over precordial chest and abdominal incision site. After about one minute, the cream was wiped off the animal with dry gauze, and finally wiped with warm saline soaked gauze to prevent skin irritation. An electrocardiogram (ECG) was obtained by applying electrolyte gel to all four paws of the rat and secured with adhesive tape. A temperature probe was inserted rectally, to monitor any loss of temperature during the procedure. A 3mm layer of ultrasound conducting gel was applied on the prepared area for ultrasonography. Ultrasound studies were conducted with a linear MS 250 probe (Fujifilm VisualSonics ®, Toronto, Canada) with a resolution ranging from 13-24 MHz, using the Vevo 2100 ® (Fujifilm VisualSonics ®, Toronto, Canada) with a 30 micron resolution and 740 fps.

2.5 Image Acquisition

The probe was held at 90 degrees to the abdominal surface of the rat, traversing the aorta in a set of sweep images obtained from cranial to caudal over the abdominal incision site. The images of the aorta in transverse view and its branches serving as landmarks were acquired, optimized and captured for measurement of aortic diameter. The probe was then rotated clockwise 90 degrees for measurement of velocity in the long axis. To optimize the image, maximized colour and power gain was used in greyscale, colour and spectral modalities to avoid aliasing or background noise. Under these standard conditions, if any inadequacies of flow measurements were present, further alterations were made to gain quality. To visualize adequate 2D images of the ventral and dorsal walls of the aorta and the vascular graft, the gain was adjusted accordingly. The walls of the graft and vessels was depicted by Doppler colour flow inside the graft with an echo/free space inside the graft, indicating patency. Pulse wave Doppler velocity was conducted by selecting an angle of insonation less than 60 degrees, this was to minimize error between the true velocity and measured velocity as related to the cosine of the angle of insonation. The proximal and distal aortic flow velocity was then measured with pulse wave Doppler, proximal and distal aortic diameter measured in M-mode and presence and type of flow (triphasic/biphasic/monophasic) was assessed with pulse wave Doppler ultrasound evaluation and recorded.

On completion of the investigation, US gel was wiped from abdomen and the animals were placed back in their cages and monitored in a warm environment until awake and ambulating. Immediate termination of the study, with graft explant, was performed if there were any features suggestive of graft occlusion during ultrasound. A narrowed lumen of the graft or aorta at the level of the anastomoses, in the absence or presence of turbulent colour flow on Doppler evaluation, was defined as a stenosis. The presence of a thrombus in the graft lumen, depicted as a large 2D hyperechoic lesion on ultrasonography, without the presence of flow on Colour Doppler was defined as an obstructed graft. The graft was considered to be occluded if there was no colour flow detected in the graft, no distal lumen present and in the absence of triphasic flow distally. The study was immediately terminated, irrespective of duration of implantation, if any of the above features were present. If the graft was deemed patent on Doppler ultrasonography, the study was only terminated at 12 weeks of implantation or in an event of a humane endpoint.

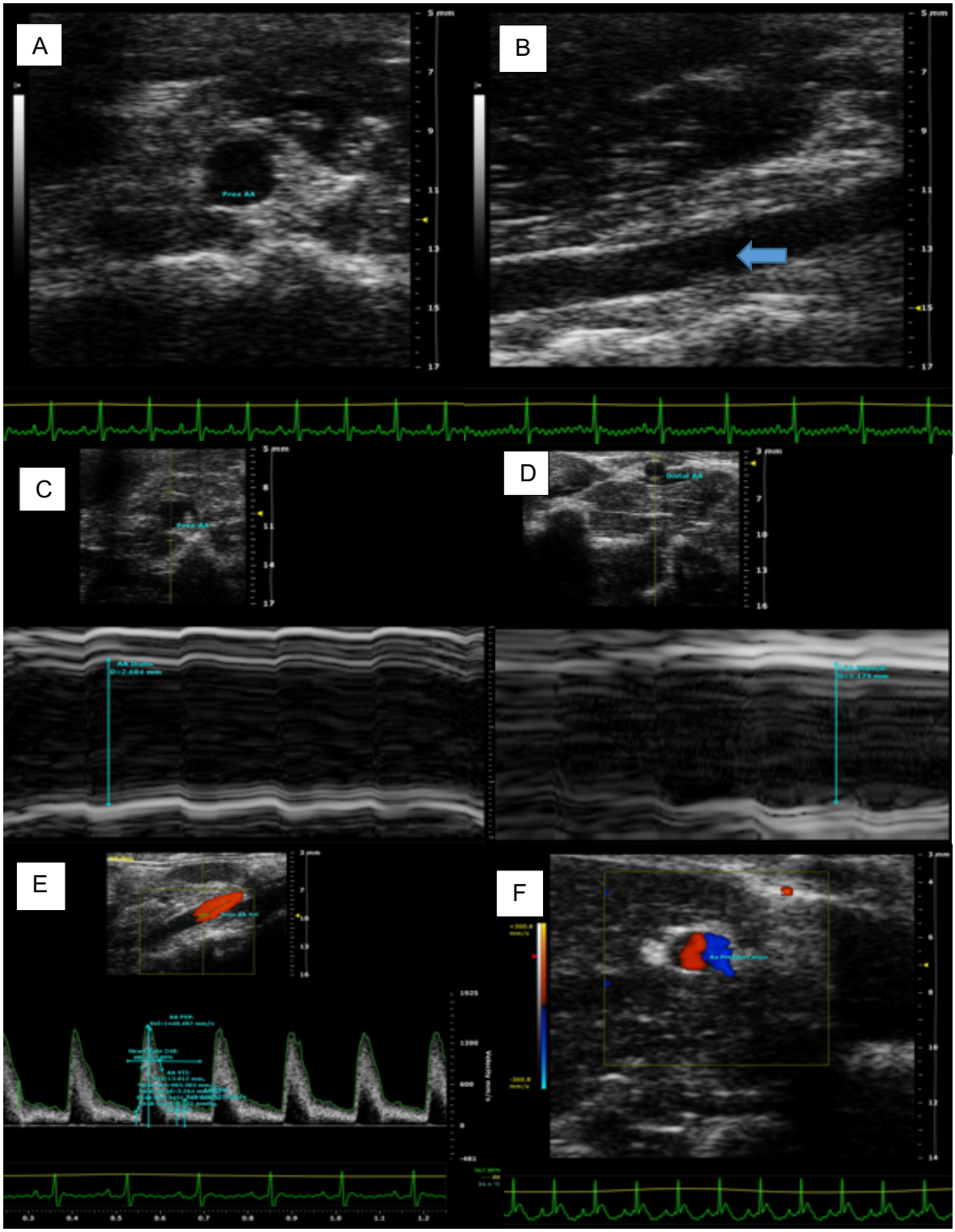

Figure 4: Ultrasonography of Grafts_*(A) and (B) showing the cross section and the longitudinal section with graft anastomoses, of the proximal abdominal aorta, respectively. (C) and (D) indicating the cross sectional diameter of the lumen of proximal and distal aorta measured in M-Mode, respectively. (E) The proximal and distal aortic flow velocity measured with pulse wave Doppler and the presence and type of flow (triphasic) assessed with colour and pulse wave Doppler ultrasound evaluation. (F) Showing distal abdominal aortic colour Doppler flow of the aortic bifurcation into iliac arteries.*

2.6 Echocardiography

Standard assessment of cardiac parameters and function were mainly acquired from the Parasternal Long Axis View (PSLAX) and Mid-papillary Short Axis View. (SAX). An ejection fraction of the heart was assessed in these views and calculated in M-Mode.

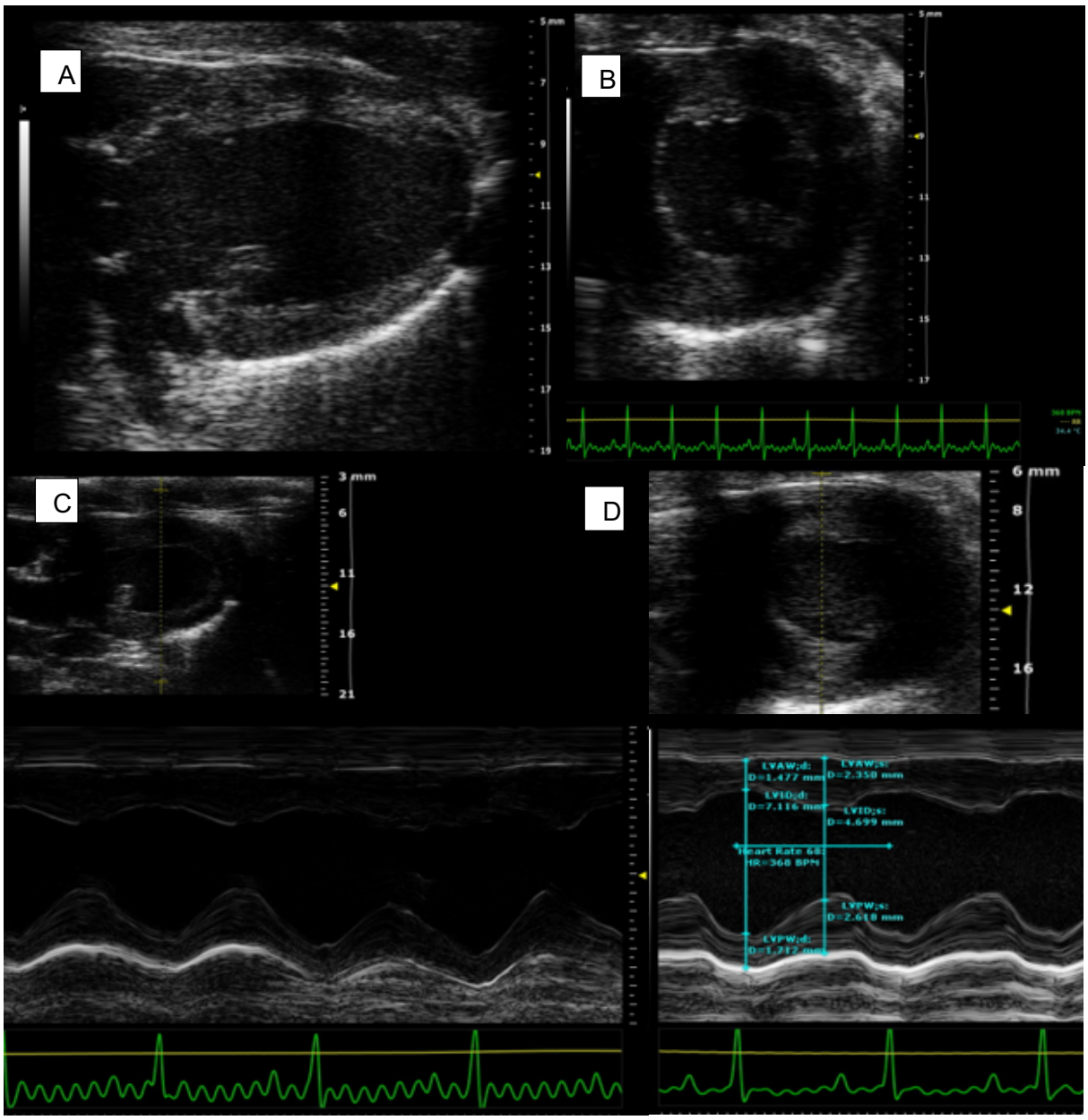

Figure 5: Echocardiography Rat Heart *(A) and (B) Parasternal Long Axis (PSLAX) view and Mid-papillary Short Axis (SAX) view of the rat heart, respectively. (C) and (D) showing an ejection fraction (EF) of the heart performed in PSLAX and SAX views, respectively and calculated in M-Mode. (LVAW;d, Left Ventricular Anterior Wall in diastole, LVAW;s, Left Ventricular Anterior Wall in systole, LVPW;d, Left Ventricular Posterior Wall in diastole, LVPW;s, Left Ventricular Posterior Wall in systole, LVID;d, Left Ventricular Internal Diameter in diastole, LVID;s, Left Ventricular Internal Diameter in systole, HR, heart rate.)*

2.7 Angiography and Explant of Infra-Renal Graft

After 12 weeks or on detection of occlusion of the interposition graft with Doppler ultrasonography, the experiment was terminated (earlier if humane endpoints were reached). Under general anaesthesia, the last Doppler ultrasonographic evaluation of graft was done to determine degree of patency/obstruction of the graft. Explant of the vascular graft was performed under Isoflurane anaesthesia (as mentioned above). Re-entry of the abdomen was via previous laparotomy. Visual patency test of distal aorta was performed and documented, followed by a dose of heparin (1mg/kg) injected into the inferior vena cava (IVC) before the animal was euthanised.

Laparotomy was extended into a sternotomy to expose the heart. An incision was made into the right atrium and the animal was exsanguinated. On apnoea, a 22-gauge cannula was advanced trans-apically, via the left ventricle, into the ascending aorta and a single dose of 5ml iodine contrast solution was given. A time-controlled x-ray was taken with a C-arm (Phillips® BV Pulsera, Massachusetts, US) while injecting contrast medium, which was followed through until contrast reached caudal and femoral vessels. Once angiography was complete, a phosphate buffered solution was introduced via the same cannula in the apex of the heart, to flush the abdominal aorta and graft. This process was continued until blood was replaced by clear fluid draining out of the right atrium. Following this, the graft underwent perfusion-fixation with 150ml of a 10% formalin solution, and was next excised and sectioned to be processed for histology.

2.8 Histological Processing

The 18 mm ePTFE straight grafts were cut with a 5mm longitudinal segment at proximal and distal anastomoses and a 5mm transverse midsection through centre of the graft. The loop grafts in turn were cut into 5 segments. This included a 5mm longitudinal segment at proximal and distal anastomoses. Two 5mm transverse segments proximal and distal to the mid-graft polyurethane segment and a 5mm transverse segment through the centre of the mid-graft segment were cut. The graft segments were all fixed in 10% Formalin for histological assessments. The samples were sliced into 6µm sections and Haematoxylin Eosin and Miller and Masson's trichrome stains were performed on these sections for basic light histological analysis. The vascular conduits underwent histological processing to assess the presence of obstruction of the vascular grafts and results were correlated with ultrasonographic findings.

2.9 Statistical Analysis

All data was imported into JMP statistical software (version 11.0, Cary; NC) for statistical analysis. Results were expressed as mean ± standard deviation (SD) for continuous variables. Following a Shapiro-Wilk test for normality, the unpaired t-test was used to compare normally distributed data and Mann-Whitney for nonparametric values. The level of significance was set at $P < 0.05$. Graphical representation used a lambda smoothing function (0.01) to describe the relationship of the scatter plot. A smooth curve through the scatter was generated with cubic splicing

3. Results

Infra renal vascular grafts were implanted in 20 Wistar rats. Group 1 consisted of straight ePTFE grafts implanted in the infra-renal aorta of 8 rats. Group 2 had loop grafts implanted in twelve rats. Graft patency was evaluated with ultrasound on day 1, 3 and 7 as well as 4, 8 and 12 weeks post-operatively.

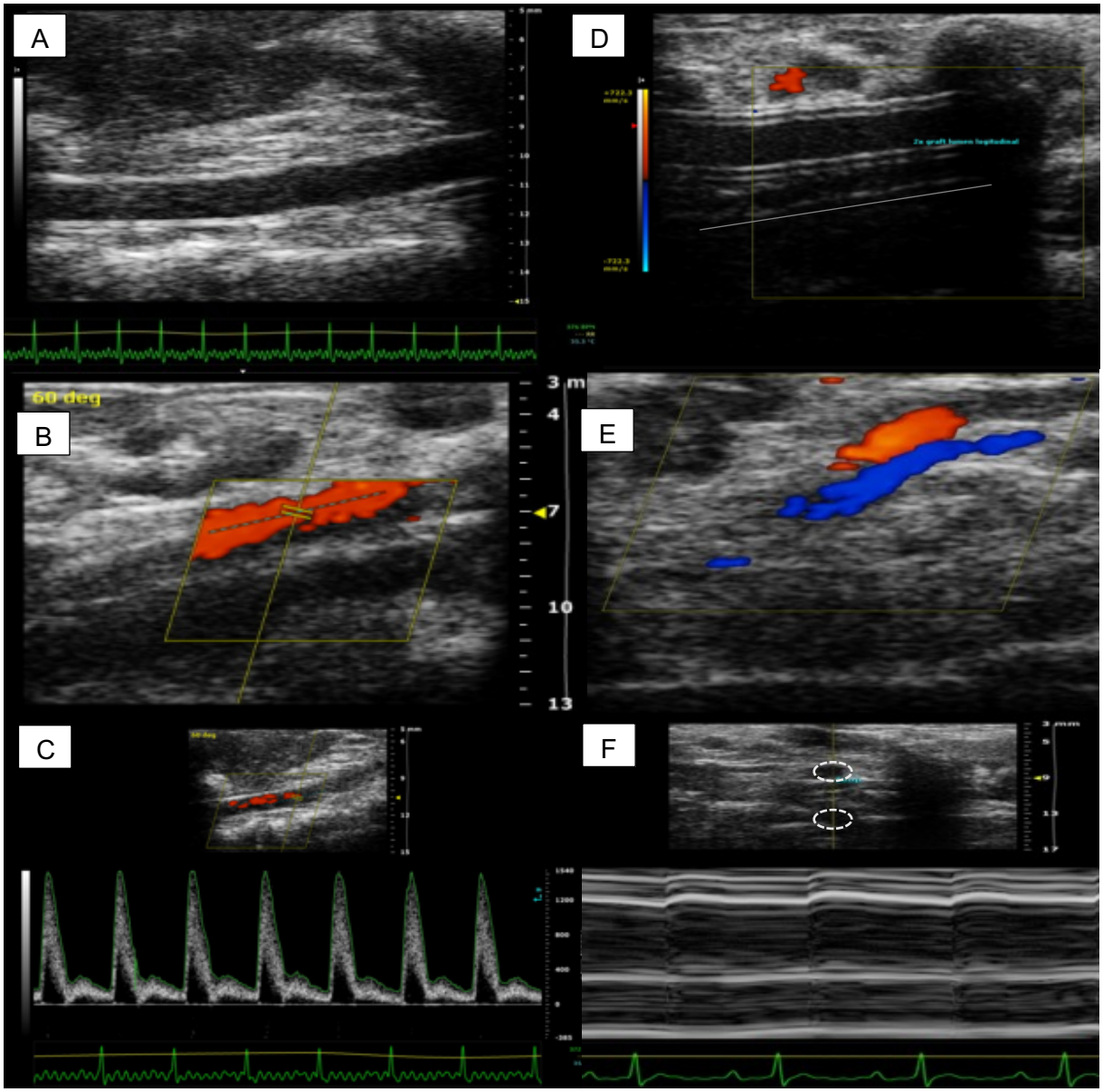

Figure 6: Ultrasound Comparison of Straight verses Looped Grafts *(A) Longitudinal section in M-Mode with the entire length of straight graft visualized in a single frame. (B) Colour Doppler flow through straight graft. (C) Calculation of flow velocity in straight graft using Pulse Wave Doppler modes. (D) Shows the longitudinal section through both arms of the loop graft in the centre of the graft where both arms cross over. The entire loop graft being too large to visualize in a single frame. (E) Colour Doppler indicating bidirectional flow in both arms of the loop graft. (F) Cross section through center of the loop graft showing both lumens of the graft.*

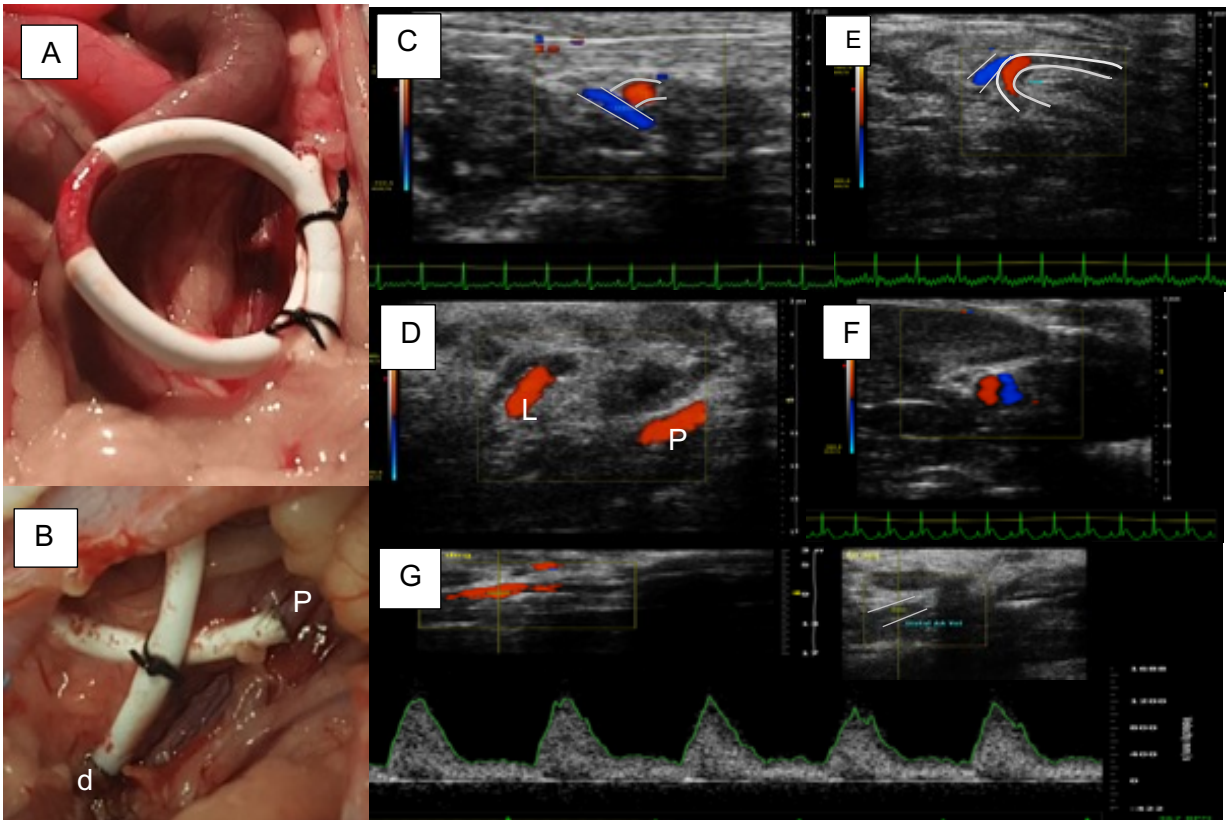

Figure 7: Ultrasound Investigation of Patency in Loop Vascular Grafts *(A) Loop Graft post implantation. (B) Loop Graft just before explant, with proximal (p) and distal (d) anastomoses visible (C) Colour Doppler indicating the centre of the loop graft with flow in opposite direction in each arm. (D) Colour Doppler flow in proximal arm of loop (P) and flow in center of the loop (L). (E) Part of the loop graft visualized on colour Doppler with distal arm of graft having flow in the opposite direction. (F) Colour flow in both lumens of loop graft lying side by side, in cross section. (G) Pulse Wave Doppler to determine the velocity in both proximal and distal arms of loop graft.*

In Group 1, Doppler had a 100% sensitivity for detecting patency and angiography corresponded with the ultrasound Doppler findings confirming all grafts patent at the twelve-week explant.

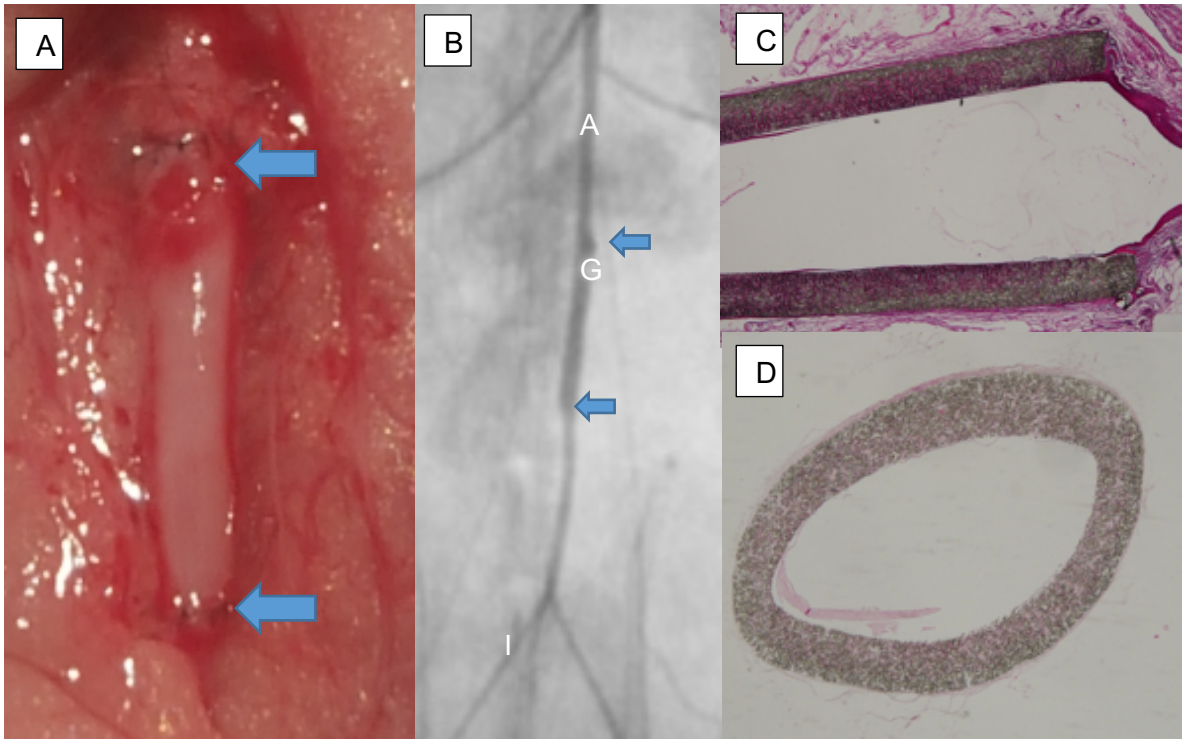

Figure 8: Straight Graft Angiography and Histology Results *(A) Straight ePTFE graft at 12 weeks implantation with arrows indicating proximal and distal anastomoses, respectively. (B) Angiogram with contrast indicating patent straight ePTFE graft at explantation, with arrows indicating proximal and distal anastomoses. (C) And (D) histological samples of the longitudinal and cross section of a straight ePTFE graft, respectively, indicating a patent lumen in both sections. (G, Graft; A, Aorta; I, Iliac Artery).*

In Group 2 at explant, 9 of the twelve grafts were occluded when compared with angiography. Ultrasonography correctly identified 7 of the occluded looped grafts, of which 3 were correctly explanted early at 8 weeks. Two of the nine grafts were, however, incorrectly identified as patent by Doppler but not angiography at 12 weeks (2 false positives).

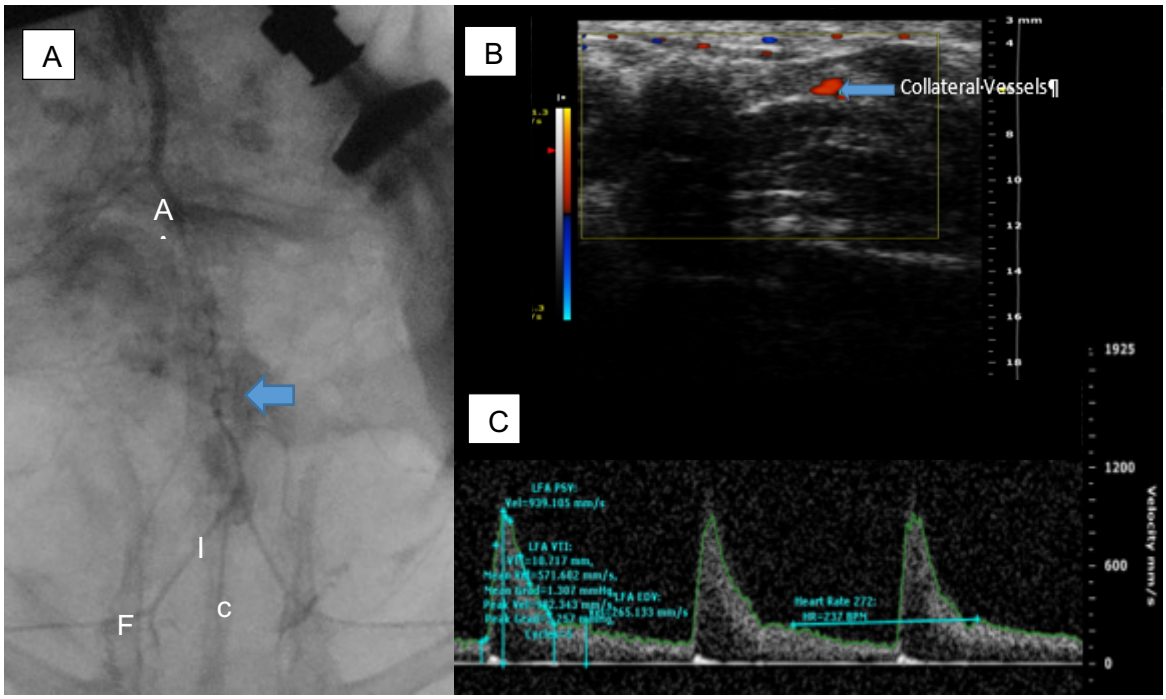

Figure 9: Obstructed Loop Graft Angiography and Ultrasonography (First False Positive Result) *(A) Angiography of occluded loop graft incorrectly thought to be patent. Arrow shows an absence of the infra-renal loop graft conduit due to occlusion, with a dense network of collateral vessels and a large filling vessel (arrow) supplying distal iliac (I), femoral (F) and caudal arteries (c). (B) Arrow indicating presence of multiple large and small collateral vessels, with lumen of graft obstructed. (C) During assessment of patency at 12 weeks with Pulse wave Doppler, both triphasic flow with measurable velocity could be detected.*

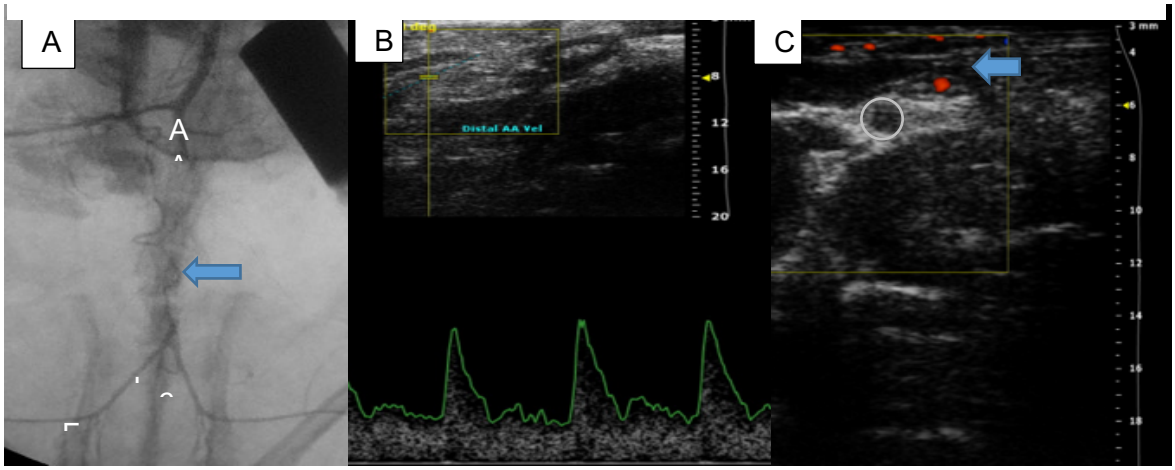

Figure 10: Obstructed Loop Graft Angiography and Ultrasonography (Second False Positive Result) *(A) Angiography of occluded loop graft incorrectly thought to be patent, with a large collateral vessel (arrow). Vessel supplying good flow to distal Iliac (I), Femoral (F) and Caudal (c) arteries. (B) Pulse wave Doppler with measurable velocity with triphasic flow. (C) Large collateral vessels seen on colour Doppler with no flow in distal lumen of graft.*

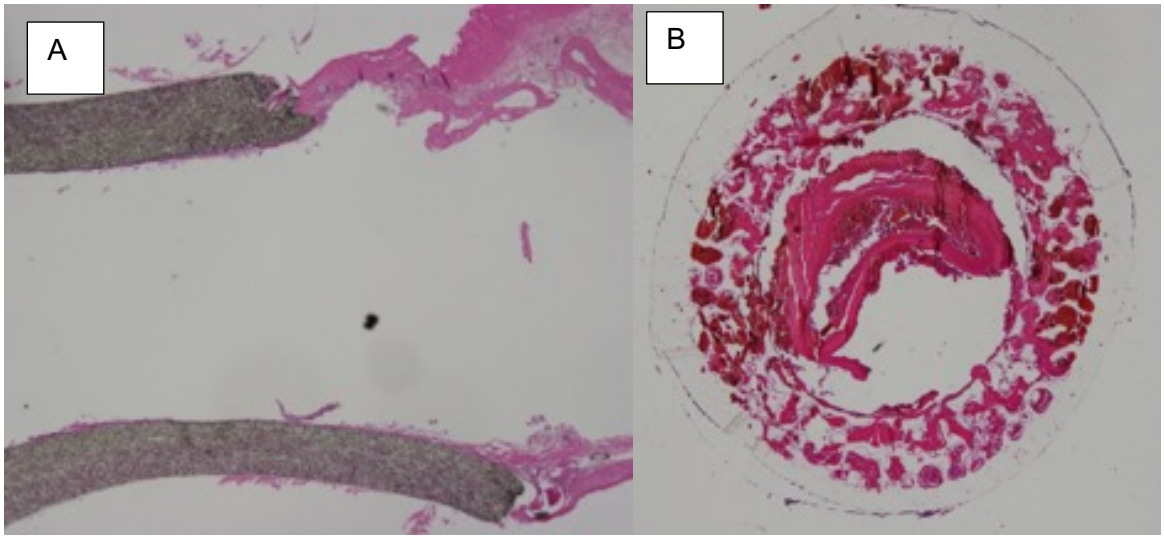

Figure 11: Loop Graft Histology *Histological sections in 2% glutaraldehyde embedded in paraffin and sliced into 6µm in thickness with Haematoxyline Eosin and Miller and Masson's trichrome stain. (A) Proximal 5mm longitudinal segment of looped vascular graft, with patency of the lumen evident on histology. (B) Cross sectional mid-graft segment of the same graft indicating obstruction of the lumen. Histological sections indicate difficulties in assessing patency of loop graft segments with some sections being patent and others partially occluded.*

The remaining 3 of the 12 loop grafts were explanted early for clinical reasons. Of these patent loop grafts, one rat had an embolic event to the caudal artery, which resulted in ischaemia of the tail tip, but Doppler still showed patency of the looped vascular graft. Two animals were euthanised for humane endpoints before 12 weeks; and in these three animals the grafts were patent by both Doppler ultrasonography and angiography.

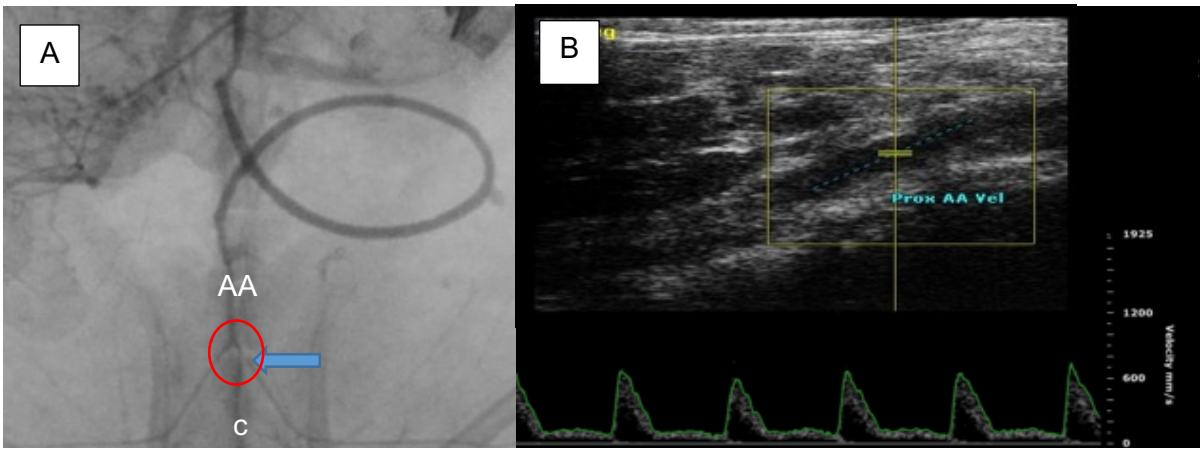

Figure 12 : Patent Loop Graft *(A) Angiography of a patent loop graft in infra-renal abdominal aorta (AA) with an arrow indicating a proximal filling defect in caudal (c) artery possibly leading to distal embolism and tail necrosis. (B) Pulse wave Doppler, detecting proximal and distal velocity in the loop graft confirming patency of graft.* Indicating patent lumen with triphasic laminar flow during pulse wave Doppler.(Vel Velocity)

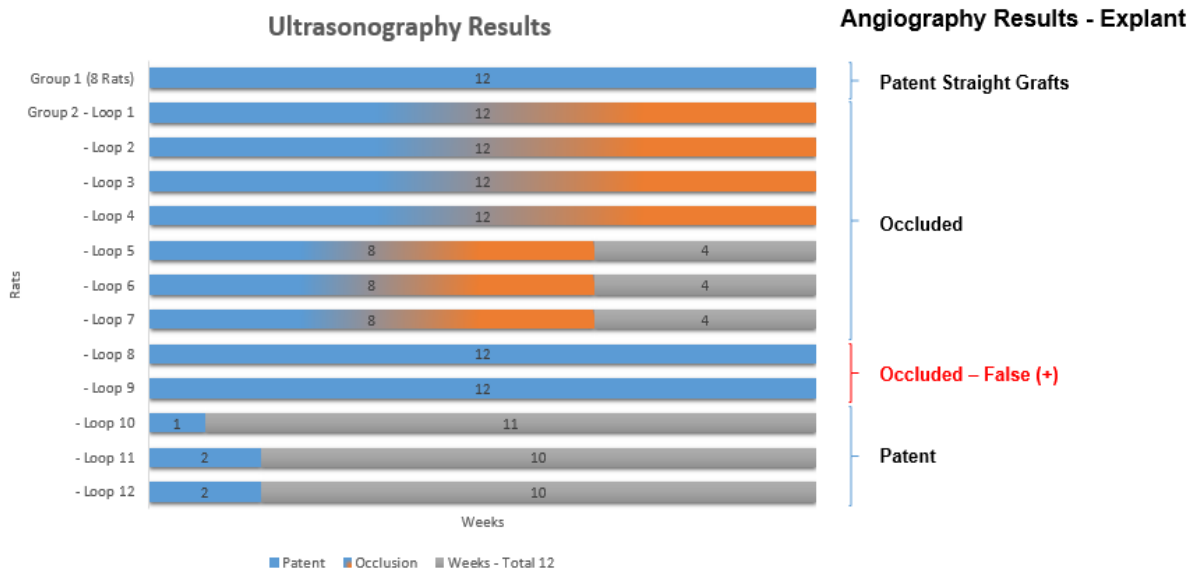

Figure 13: Graphical Presentation of Study Results *Comparison of ultrasonographic findings of Group 1 and 2 with angiographic findings at explantation at 12 weeks.*

Doppler had a 100% specificity and 78% sensitivity to detect occlusion in the 20 grafts implanted. The results between ultrasound and angiography corresponded in 18/20 animals that had implants performed with a positive predictive value (PPV) calculated at a 100% and negative predictive value (NPV) of 85%.

Results Positive: Grafts Occluded on Ultrasound Evaluation

	Positive	Negative	Measures
Positive	True Positive **7**	False Positive **0**	Positive Predictive Value **100%**
Negative	False Negative **2**	True Negative **11**	Negative Predictive Value **85%**
Measures	Sensitivity **78%**	Specificity **100%**	**N=20**

Figure 14: Statistical Presentation of Findings of Ultrasound *Ability of ultrasound to detect occlusion of implanted straight and looped infra-renal vascular grafts.*

Miscellaneous Ultrasound and Echocardiography Findings

Initially ultrasonography evaluation of graft patency was performed directly post surgery. Imaging indicated an empty left ventricle indicative of significant hypovolaemia

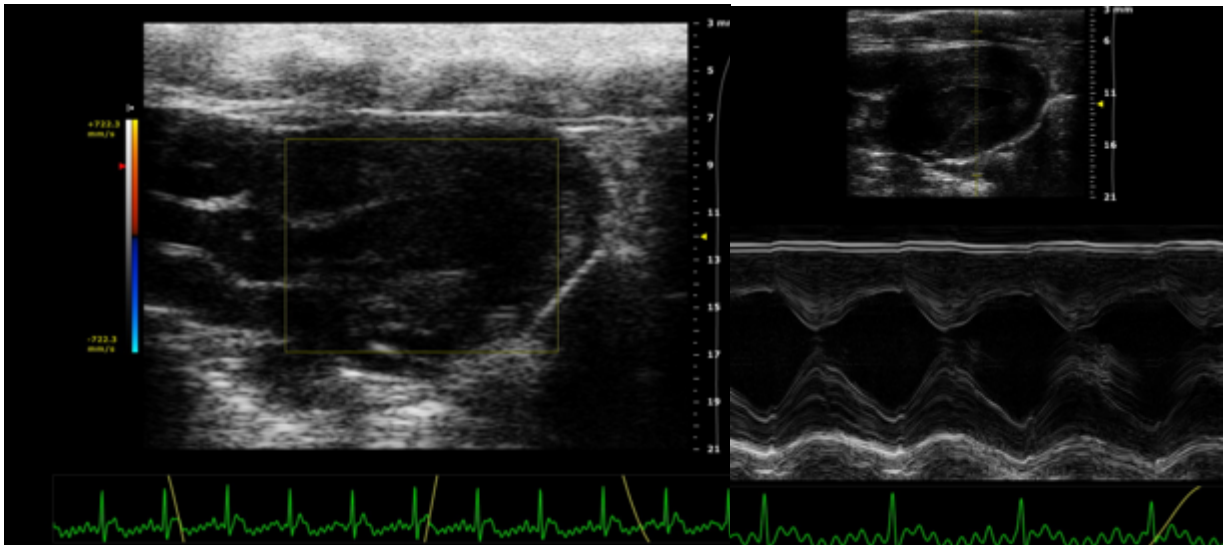

Figure 15: Left Ventricle Post-Operative Hypovolaemia *Echocardiography of parasternal long-axis view indicating poor filling of the left ventricle of rat directly post operatively with features evident of hypovolaemia on imaging*

Injury of the iliolumbar and sciatic nerves during clamping of the iliolumbar vein lead to a neuropraxia in two of the rats. Ultrasound confirmed that neurological fallout, presenting as a unilateral leg lag of the hind leg, was not due to an ischaemic event. Ultrasound indicated good distal flow from aortic bifurcation to femoral arteries.

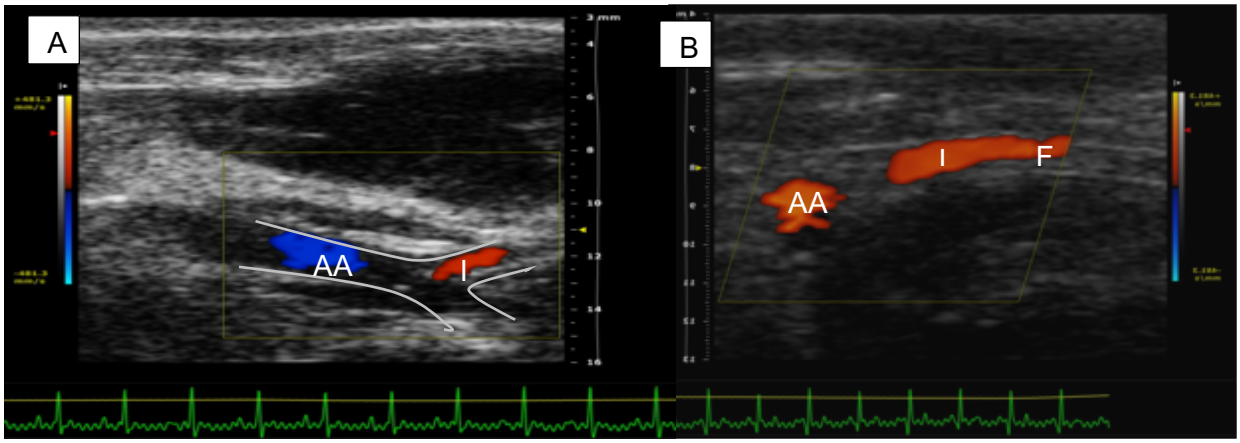

Figure 16: Right Neuropraxia Due to Iliolumbar Nerve Injury *(A) Colour Doppler, indicating aortic bifurcation into iliac vessels and (B) flow down the left femoral artery in an animal with leg lag of left hind leg. Confirming that no ischaemia is present, neurological injury due to Neuropraxia of the nerve and not due to an ischaemic event. (AA abdominal aorta, I Iliac artery, F femoral artery)*

Echocardiography was performed to determine the ejection fraction (EF) of left ventricle (LV) compared between straight and loop grafts at different time points, but was not found to have statistical significance.

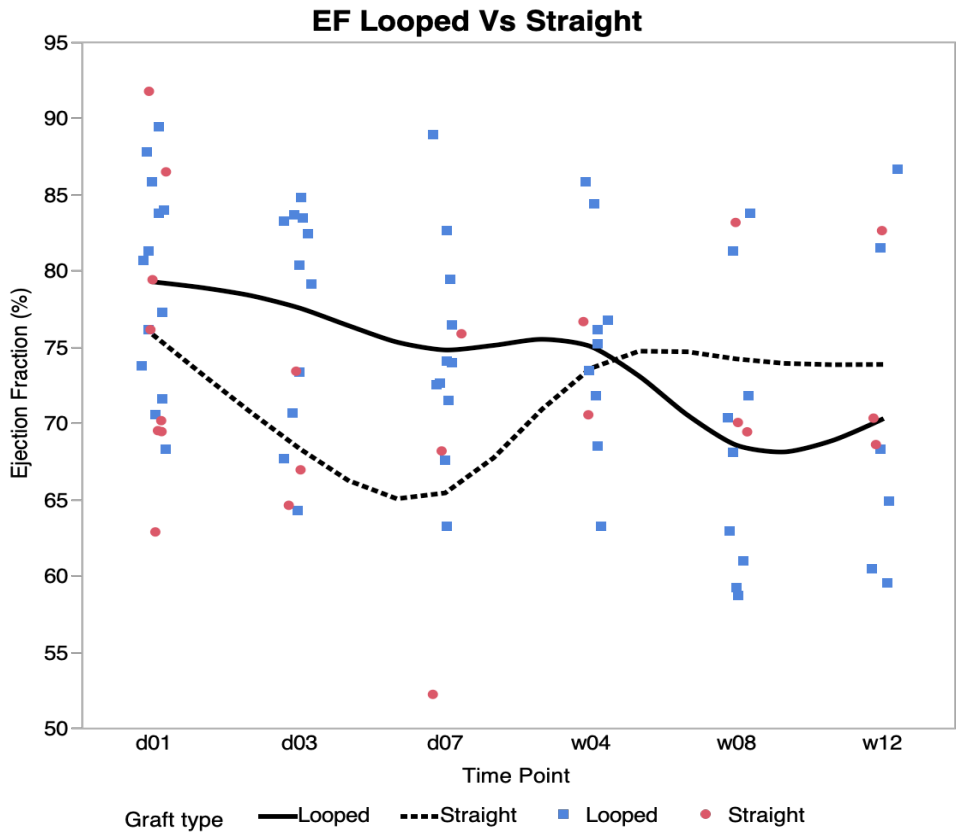

Figure 17: Parasternal Long Axis Ejection Fraction Comparison in Straight and Loop Grafts *Graft showing the ejection fraction in both the loop and straight grafts, with an initial decrease in ejection fraction in the first week. Normalization of the ejection fraction back to baseline before explant. (p=ns) (EF – Ejection Fraction).*

Evaluation of EF of preoperative control group and post-operative EF in Group 1, did not reveal any statistical significance. Similar findings were found when comparing aortic diameter and peak and mean systolic aortic velocities. None of the results of these variables held any statistical significance, but the results did indicate an upward trend in specific values of these variables over time and are thus included below. Weight gain between male and female rats in the straight graft (Group 1) did reveal statistical significance.

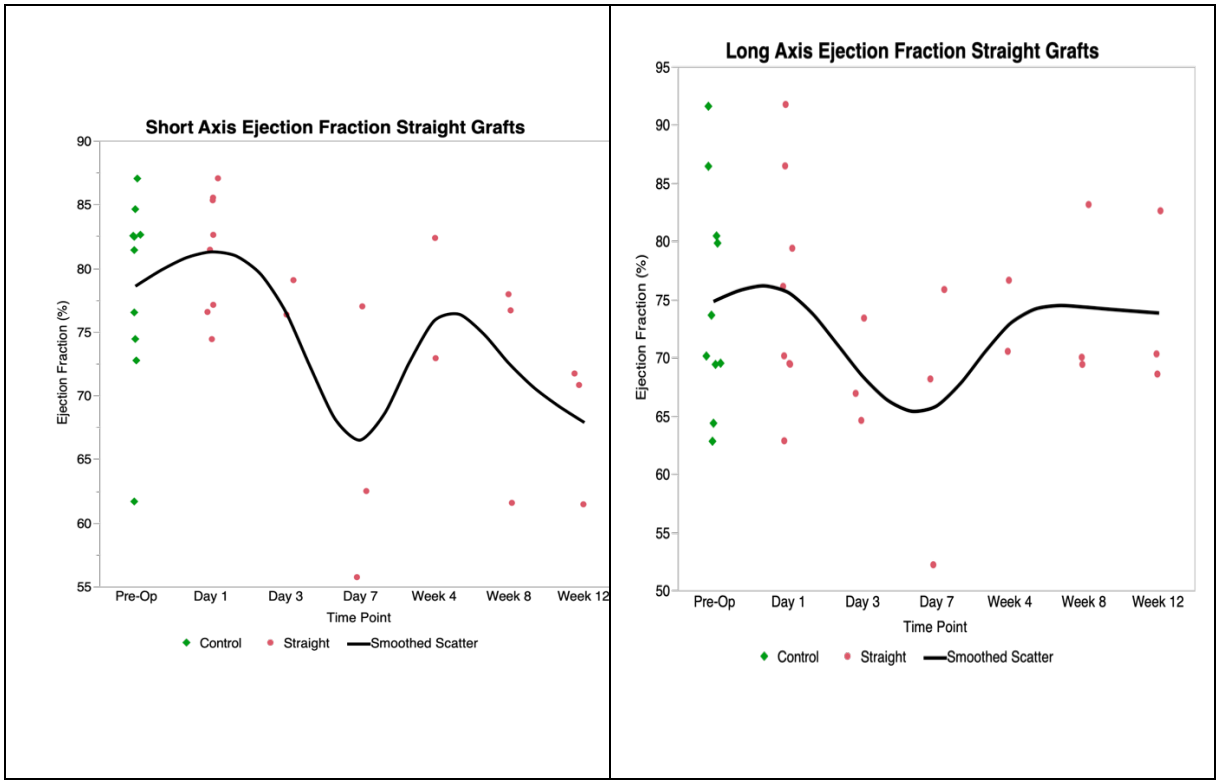

Figure 18: Short Axis and Parasternal Long Axis Ejection Fraction *Comparison between preoperative control group and post-operative straight grafts group (p=ns)*

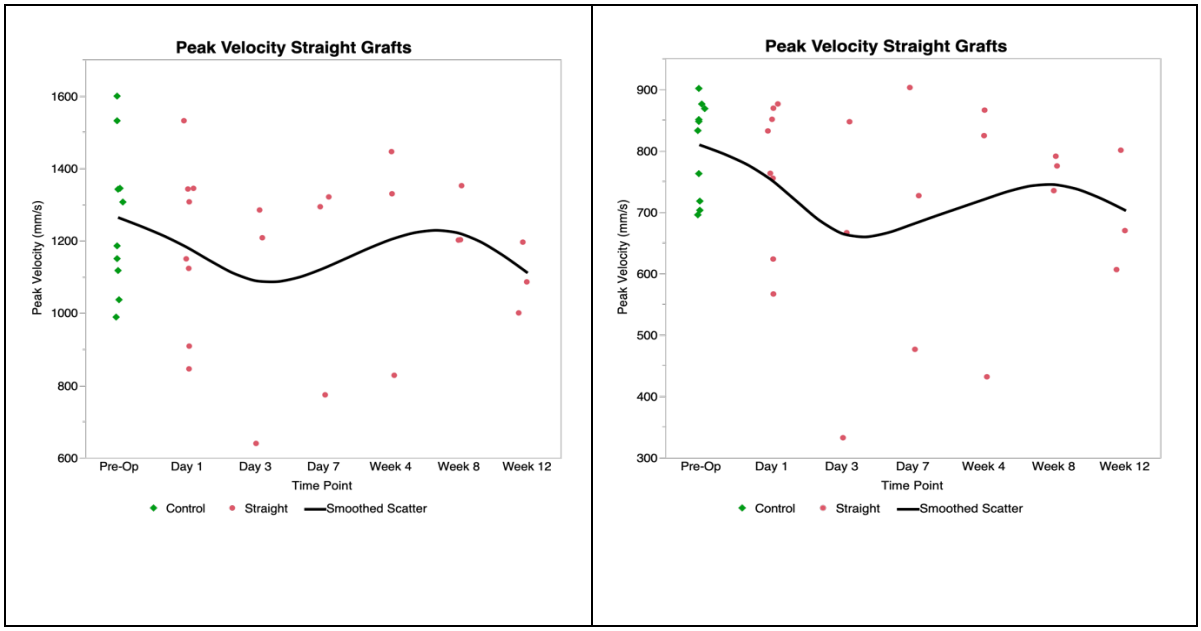

Figure 19: Aortic Peak and Mean Systolic Velocity Comparison between pre-operative control group and post-operative straight graft group measurements (p=ns)

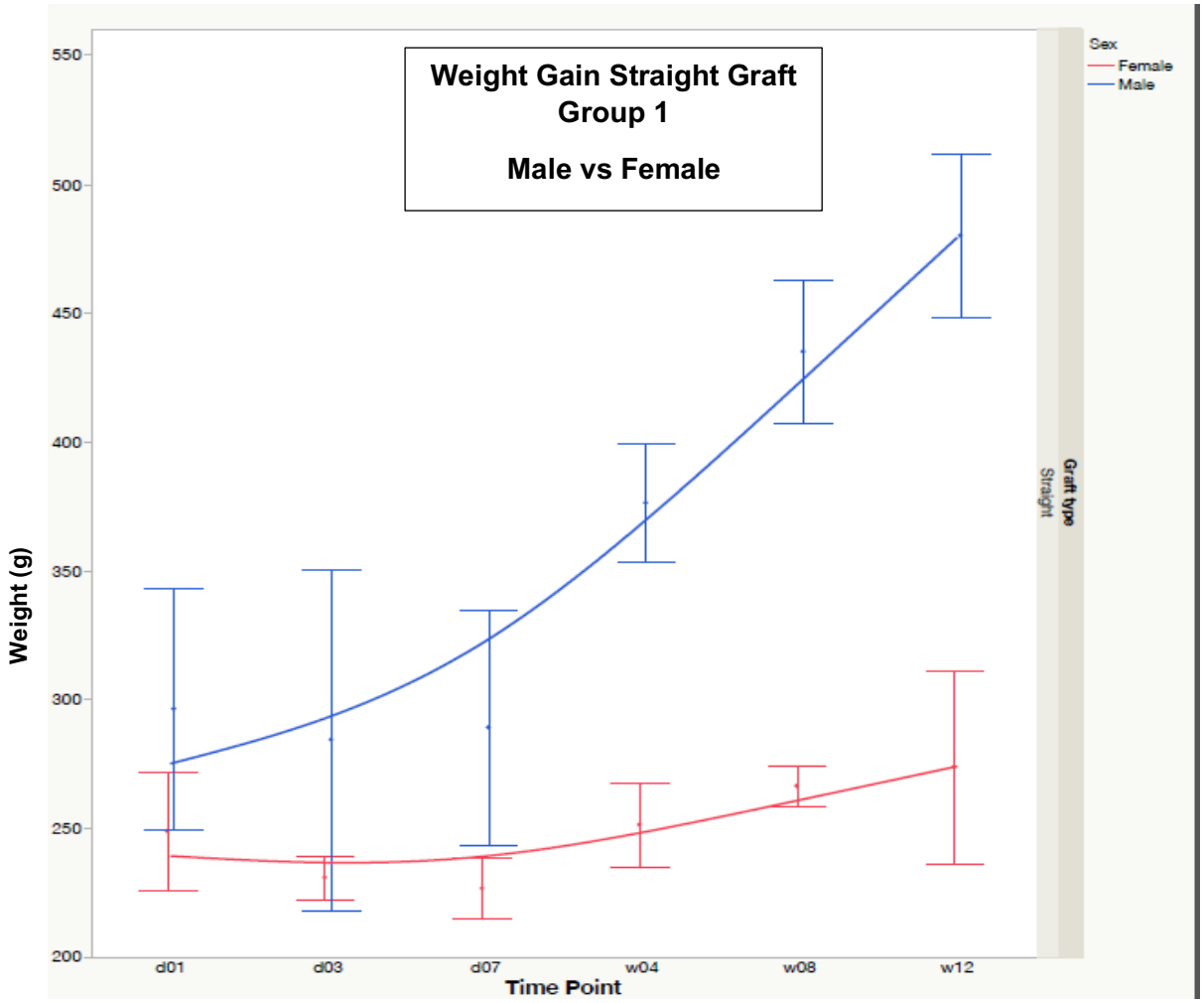

Figure 20: Weight Gain in Straight Graft Group 1 *Comparison of weight gain in Group 1 male versus female rats. (p <0.05)*

4. Discussion

Chronic follow up and long-term review of implanted experimental grafts becomes crucial to the success and progress of vascular research. Evaluation of graft patency by means of angiography, considered to be the gold standard, can lead to increased morbidity as this requires vascular cannulation and injection of a relatively large volume of contrast, which in turn could have detrimental outcomes in small animal models.(7, 11) After review of multiple vascular graft studies, very few research articles elaborate on the means and effectiveness of the non-invasive evaluation of patency and occlusion of vascular grafts in small animal models.(9, 11, 17-23) It was therefore proposed that ultrasound could be used as a modality in evaluating patency of implanted vascular grafts, and lead to the refinement of our animal research. We applied vascular ultrasound at six different time points post operatively, namely at day 1, 3, 7 and week 4, 8 and 12 to detect occlusion.

All the straight grafts were easily detected as patent *(Figure 8)* at all time points, which was confirmed by angiography and histology at 12 weeks. The sensitivity was high but specificity of only 78% was found with a negative predictive value of 85% *(Figure 14).* In similar research conducted in large animal models, inaccuracy of Doppler findings seemed to be less of a problem. Doppler ultrasonography was found to be more accurate when used to evaluate patency or occlusion of implants in carotid arteries in pigs at different time frames, compared with angiography.(18) At four week follow up the NPV was still as high as 95%, PPV of 92%, sensitivity of 86% specificity of 92% in determining patency of carotid grafts in pigs.(18)

The difficulties in conducting ultrasound evaluation in small animals versus large animals are clearly defined.(3, 24) In large animals ultrasonographic imaging of tissue is limited to a depth of 25cm.(24) In small animals the depth of imaging is less of a problem but difficulties result in separating various tissue planes. Higher frequency probes are required to improve spatial and temporal resolution.(3) Faster heart rates, the need for anaesthesia and faster reduction in body temperatures, which may result in haemodynamic instability during ultrasonography, also present additional difficulties in small animals. (10)

The results found in our study *(Figure 13),* could be explained by the fact that the straight grafts could easily be evaluated in the small animal model since the entire length of the straight graft could be visualized during ultrasound evaluation *(Figure 6)*. Visualizing the entire loop graft during ultrasound was technically more difficult. Only the central structure of the graft, which consisted of the proximal anastomotic segment, the crossover (central) segment with bidirectional flow and the distal anastomotic segment of the loop graft could be assessed in

separate frames, due to the size and angles of the graft. Although the entire looped graft could not be visualized in a single frame, we accepted that if we could detect flow, and flow related parameters this would be adequate to determine loop graft patency *(Figure 7)*.

This study emphasized the importance of being able to accurately assess patency of implanted graft material at any time frame. Studies have shown the ability of Doppler ultrasonography to detect patency in simple graft models, but difficulties do arise in detecting occlusion or absence of flow.(7, 25) It is important to determine the parameters that define patency of vascular implants. Patency may be described using four descriptive modalities, namely: [1] Clinical modalities, [2] In vivo non-invasive modality by means of ultrasonography, [3] In-vivo invasive modality by means of angiography and [4] Ex-vivo modality by means of histological assessment.

The first modality pertains to the macroscopic clinical evaluation of the graft during implantation. This is not well described in vascular graft literature.(14, 26) The graft should be considered patent if strongly pulsatile and good flow can be observed with distention of the distal aorta under vision after completion of infra-renal implantation and needs to be present at the time of explant.(14, 26) Patency may also be assumed if in the acute post-operative period, the animal has normal motor function of tail and hind legs.(26) The latter clinical parameter does not hold true in chronic implantation models, since slow occlusion of the vessels may lead to collateralization *(Figure 10),* which would result in adequate perfusion leading to an absence of any motor fallout, even in complete occlusion of the graft.

The second modality is the in-vivo non-invasive functional assessment of patency with ultrasonography, which has been described as detecting femoral arterial flow on Doppler imaging.(26) The development of new ultrasound modalities with higher frequency and thus resolutions, have made it possible to accurately assess flow in smaller blood vessels.(27) Use of laser Doppler modalities to measure superficial blood flow, is now routinely used, including the ability to detect flow in femoral arteries.(26) But distal superficial flow may arise from collaterals. Studies implementing Laser Doppler measurements, have looked at the flow in the hind limbs of rabbits after implantation of multilevel occluded conduits in femoral arteries.(28) Doppler flow indicated surface perfusion, which was contributed by blood flow from collateral vessels that formed due to occlusion of the graft. (28)

As noted in our study, it is not appropriate to assume that pulsatile blood flow through femoral arteries translates into patency of aortic vascular grafts. Our results confirm that even in the presence of complete occlusion of the vascular graft, formation of collateral vessels can be so extensive that they allow for complete distal femoral arterial flow without any clinical signs of

motor function fallout in the animal *(Figure 9)*. Collateral vessels may also be so large that they mimic the same calibre and velocity on Doppler ultrasonography, as aortic flow or flow through the patent graft *(Figure 10)*. This finding is particularly relevant in small rodent models such as mice and rats with small calibre grafts.

Doppler ultrasonography is considered an established method of detecting blood flow. However, due to the one dimensional nature of this measurement, there have been concerns with regards to its accuracy.(7) The velocity of blood flow on spectral Doppler is only captured in the same direction as the ultrasound beam, with an estimation of the accurate direction of the blood flow.(7) Angle correction is essential to direct the ultrasound beam in an accurate direction to flow, so that reliable quantitative velocity measurements will be measured.(2, 7) Four parameters need to be met using different modes of ultrasonography to assess the functional modality in the evaluation of graft patency. The graft was recorded as patent under a non-invasive modality if the following four criteria were met: [1] The distal lumen of the graft could be measured, [2] colour Doppler flow was present in the lumen, [3] measurable distal velocity with a triphasic flow pattern and [4] no hyperechoic density could be detected in the lumen.

There were, however, two false positives in the loop graft group, *(Figure 9 and Figure 10)* where the grafts were considered to be patent on Doppler ultrasonography evaluation at twelve weeks of implantation with all the above parameters present. In both cases the grafts were found to be occluded on angiography with indeterminate results on histological evaluation *(Figure 11).* In these two grafts, it was difficult to measure the distal lumen diameter, however the distal velocity was obtained with colour and triphasic flow present on Doppler. Both the Ultrasonographers felt that there were significant features suggestive of patency of these two grafts. All the above parameters were absent in the other seven loop graft conduits which were correctly detected as occluded on ultrasound evaluation and confirmed occluded on angiography at explant *(Figure 13).* On review of the ultrasound, with the hindsight of angiographic evidence, it was evident that the misinterpretation of flow (colour Doppler and triphasic flow) was due to a dense network of large tortuous collateral vessels supplying the distal aorta.

The third modality of determining graft patency is angiography, which is invasive and occurs at explant. The fourth modality is a histological assessment of graft patency once explanted ex-vivo. Although the gold standard in determining vascular graft patency is still considered to be angiography,(29) histology is often used in vascular graft studies as a surrogate to assess graft patency.(14, 30) In the short straight grafts, the histology correlated well with the ultrasound and angiographic findings, however in the longer looped grafts determining

occlusion on histology was unreliable *(Figure 11).* Looped grafts are more difficult to review the entire loop histologically, and preparation of the loop graft into adequate frames is technically challenging. Segmental review of the loop graft results in possible error, since certain segments of the graft was found to be patent, where other segments were found to be occluded or partially occluded depending on region of the graft assessed. Ultimately, only angiographic results were accepted and compared to ultrasound findings.

Seven looped grafts in our study were occluded on ultrasonography and confirmed by angiography. Three of these grafts were found to be occluded by 8 weeks, reaching the primary endpoint for explant. An extensive network of collateralization was already confirmed on ultrasound as well was angiography with no contrast flow in the loop graft confirming occlusion. The three patent loop grafts were explanted earlier than the 12 week study endpoint, due to humane endpoints being reached*, (Figure 12).* Two of these animals had excessive weight loss, and the third animal had earlier termination of implantation due to necrosis of the tail tip. In all three animals ultrasonography detected patency of the loop graft and this was confirmed on angiography prior to euthanasia of the animals. A filling defect could be appreciated on Doppler ultrasonography of the caudal artery, from what was suspected to be an embolism to the tail *(Figure 12).* Doppler ultrasonography showed no occlusion of the graft itself, and patency was confirmed on angiography at explant with a filling defect of the caudal artery.

The loop graft implants were technically more difficult in female rats. Female rats had a lower weight gain compared to male rats after implantation, this was statistically significant in Group 1, the straight grafts *(Figure 20)*. Due to difficulties of implanting loop vascular grafts in female rats weighing less than 300 grams, as a result of smaller aortic size and smaller abdominal cavity to accommodate the large loop graft, fewer females were used in Group 2 and could thus not indicate a statistical significance with regards to weight gain. We would not advocate the use of female rats in straight and loop graft implantation studies in the future.

As an operator dependent investigation, there is a learning curve involved in conducting ultrasonography. All our images were confirmed by a skilled small animal Ultrasonographer and yet there was still room for error. There is a need to familiarize oneself with the various images and views when dealing with complex vascular graft models, which will help to readily detect complications in these grafts. Due to the construction of the loop graft it was impossible to visualize flow in all regions of the graft. One could only assess the regions of proximal and distal anastomoses and detect flow in the area of “cross-over” in the centre of the loop graft *(Figure 7).* We could assess the diameter, velocity and colour flow at the proximal and distal anastomoses of the loop graft with spectral Doppler ultrasonography. In patent grafts we

could detect all the above, including good laminar triphasic flow. The above parameters were obscured in the two false positive cases described where large tortuous collateral vessels were supplying distal flow. This is one of the known limitations of ultrasonography. The loop graft is a complex tortuous model and we are the only group at present doing loop graft models, which presented with the known ultrasonographic limitations. The use of ultrasonography was very effective in the straight grafts.

As an additional finding, it was noted that the ejection fraction initially decreased post operatively in both the straight and loop grafts but the ejection fraction normalized back to baseline weeks after implantation *(Figure 17).* It was speculated that the loop graft may add additional afterload to the left ventricle of the heart, leading to a compromise in ventricular function, although this was not statistically significant, the trend suggests that the longer graft may result in depressed cardiac function. This finding is however confounded by the significant difference in the patency in the two groups. During ultrasonography and echocardiography we also assessed the aortic peak and mean velocities and ejection fraction between the pre-operative control group and Group 1 (straight grafts) *(**Error! Reference source not found.** and **Error! Reference source not found.**)*, but no statistical significant changes were present in any variable in comparison to each group as depicted in the grafts displayed.

There were additional limitations to the study which included the intervals of ultrasonography post-operatively. The initial ultrasound imaging was found to be complicated by abdominal air in the early post-operative period. This made imaging difficult and sometimes did not contribute significantly or allow for adequate imaging of the graft. We also noted that the initial post-operative echocardiogram showed a very empty “kissing” left ventricle *(Figure 15),* which led to the adjustment of management with an additional fluid resuscitation given to the animals in the immediate post-operative period to improve ventricular filling. Concern was raised with regards to stability of the animals during ultrasonography due to these findings, and therefore a decision was made not to perform imaging in the immediate post-operative period. Echocardiography may however be used to determine animal volume status if required.

Clearer images were obtained from day 3 post-operatively when animals started eating and ileus subsided with adequate fluid resuscitation and better haemodynamic stability. Imaging done within 48 hours of the abdominal implantation rendered poorer images than those conducted later. We would also suggest doing weekly ultrasound evaluation of grafts instead of monthly since smaller changes attributing to occlusion of grafts could be more adequately followed on a weekly basis.

Even with the above limitations, ultrasonography allowed us to perform long-term follow up of our grafts, hence evaluation of grafts were not only limited to the day of explant. Another important use of ultrasound is the ability to review various flow parameters and soft tissue structures in the regions surrounding the surgical field. The one animal excluded from the study was euthanised after detecting a large distended bladder on ultrasonographic evaluation. The animal had cystitis and due to concerns of graft contamination and poor condition of the animal, the decision was made to euthanise the rat, and exclude the data from the study. Ultrasound therefore does not only allow for chronic follow up of vascular graft status itself, but could allow for chronic follow up that would have led to later or earlier termination of animals in the study.

Ultrasonography thus also improves the quality of research conducted. This is additionally noted during the implantation of our vascular grafts, when clamping the iliolumbar vein to inject heparin. This procedure has a risk of a neuropraxia of the iliolumbar nerve. This occurred in two animals presenting with a unilateral leg lag. Routinely these animals would have been euthanised due to concerns that the neurological fallout is due to an ischaemic event of the leg as a result of a vascular graft complication. We could, however, confirm flow in the graft and distal iliac arterial flow (*Figure 16),* excluding any acute ischaemic event and the animals were not euthanised. The Neuropraxia resolved completely within seven days post-operatively. Ultrasonography additionally allows for the review and chronic evaluation of graft flow characteristics, presence of aneurysm formation and extent of aortic dilatation, assessing parameters like vessel pulsatile flow, distensibility and stiffness.(11, 31) All information was obtained by means of chronic follow-up with ultrasonography over time.

Our study had a specificity of 100% but limitation in sensitivity to detect occlusion of 78% with PPV of 100% but NPV of 85% *(Figure 14).* This does leave some doubt with regards to detecting occlusion of the graft in more complex loop models, but it could limit angiographic evaluation to difficult cases where doubt of patency on ultrasonography is present. Ultrasonography allows for follow-up examinations to be performed with minimal consequences and discomfort to the animals or researchers. It should be seen as a modality to be used in combination with angiography to determine both the mechanism and progression of occlusion.

5. Conclusion

It is essential that during vascular graft implantation, patency may be regularly evaluated and accurately determined. Ultrasound is easily accessible providing repeatable, rapid and safe results at any time point post operatively. In our setting, the greater complexity of vascular

grafts makes imaging more difficult but considering the benefits of ultrasonography it may be worth investing in additional non-invasive imaging modalities. The complexity of imaging can be simplified with combination of more advanced non-invasive imaging modalities that complement each other and may be used to confirm findings. Newer prospective studies are required to establish optimum techniques of vascular graft imaging. We have found that this is a feasible and advantageous diagnostic technique supplying additional knowledge, at very little risk to animals that we could implement in our current vascular graft models in rodents.

6. Limitations

Study is limited by small numbers in the experimental groups. Another limitation includes the uneven division of the groups between male and female Wistar rats, due to difficulties of implantation of the loop graft model in smaller animals like the female rats, weighing less at implantation. Additional limitations to be addressed in future studies is the absence of an occlusion model in the straight graft implants. Ultrasonographic interpretation of a ligated straight graft and haemodynamic effects could yield additional findings in future studies. Consideration in future studies would be to examine flow dynamics in earlier time points in grafts at risk of occlusion, since the onset of occlusion occurred earlier than the time points reported in this study.

7. Author Contributions

Ultrasonography: NDS, NK
Angiography: NDS, NS
Surgical graft implants: NDS
Data collection: NDS
Concept and Study Design: NDS, TP, DB
Analysis of Study: NDS, TP, DB
Write up: NDS, TP, DB
Revision of article: NDS, TP, DB, PZ
Approval of article: TP
Statistical Analysis: PH, TP
Funding: TP, DB, PZ
Overall Responsibility: NDS, TP
Histological Assessments: HI, NDS

8. Disclosure Statement

No disclosures, none of the authors in the study have competing financial interests.

9. Acknowledgements

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Section 3: Future Directions

Improvements of MRI and CT imaging can provide additional information but these are expensive modalities and not readily available in our small animal research units as yet. There are multiple non-invasive modalities available including contrast induced ultrasonography and as soon as cost and funding allow, should be implemented in all small animal research units.

Information obtained in this study of the implementation of ultrasographic imaging of our units vascular grafts models, will now be incorporated in future studies. We are implementing the technique of electrospinning in fabricating biodegradable polymer vascular grafts in a PHD study. The skills and training obtained in this MMED thesis, with regards to graft implantation and ultrasonographic imaging, will now be implemented in future planned studies and the above findings will be distributed in peer reviewed journals.

Appendix

Author Instructions

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